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CYCLE

XXX

Low Protein/Gluten Free bread: special dietary uses and physical characterization.

Area of interest: ING/IND24 Principles of Chemical Engineering

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Abstract

Il presente lavoro si inquadra nel contesto dello studio di sistemi alimentari destinati ad usi dietetici speciali.

Obiettivo della ricerca è stato l'approfondimento della conoscenza di questi alimenti ed un eventuale loro miglioramento in termini sia di aspetto che di valori nutrizionali.

La ricerca è rivolta nello specifico ai prodotti da forno senza glutine. Quest'ultimi sono quelli che richiedono maggiore attenzione in quanto l'assenza di glutine genera in essi problematiche strutturali, sensoriali e nutrizionali.

Nonostante i progressi in questo campo, molti prodotti attualmente presenti sul mercato pur presentando infatti un aspetto simile a quelli tradizionali, preparati con la farina di frumento, risultano nutrizionalmente sbilanciati e spesso non soddisfano i bisogni del consumatore.

Investigando l'interazione dei vari costituenti degli alimenti, grazie allo studio delle loro proprietà chimiche, meccaniche e termiche, l'obiettivo ha riguardato il miglioramento della qualità del pane senza glutine principalmente aumentandone il contenuto in fibre, regolando il tipo e la quantità di idrocolloidi polisaccaridici (derivati dalla cellulosa) e proteici (proteine di origine vegetale) nelle formulazioni, al fine anche di contenere la quantità degli ingredienti con un conseguente beneficio in termini di costi.

Eseguendo analisi reologiche, termiche e spettroscopiche è stato possibile dimostrare che l'uso della corretta concentrazione di idrocolloidi, con un aumentato livello dell'acqua, può portare a impasti senza glutine più simili a quelli ottenuti con farine di frumento, senza l'aggiunta di ulteriori ingredienti anche se molti aspetti necessitano di essere ulteriormente migliorati.

I diversi approcci utilizzati in questo studio evidenziano che le caratteristiche macroscopiche di questo tipo di sistemi sono fortemente legate alla composizione ma soprattutto al comportamento in temperatura dei materiali considerati e ancora pochissime pubblicazioni esistono in merito a tale aspetto.

Chapter 1

LP/PF bread for special dietary use: State of Art

1.1 Introduction

Nutritional status is considered one of the primary determining factors of an individual's health and quality of life. Several diseases of our time are in fact clearly linked to lifestyle habits, particularly diet (Nestle 2003, Rissanen et al. 2003, Apovian 2004).

From a clinical point of view, nutrition is known to be a powerful tool able to slow down disease progression or improve therapeutic outcomes. In this regard, new developments are taking place in medical nutrition, a field that deals with the specific requirements of patients which have switched from a normal diet, based on the consumption of basic foods, to a medical nutrition therapy (MNT)

Figure *1*.1.
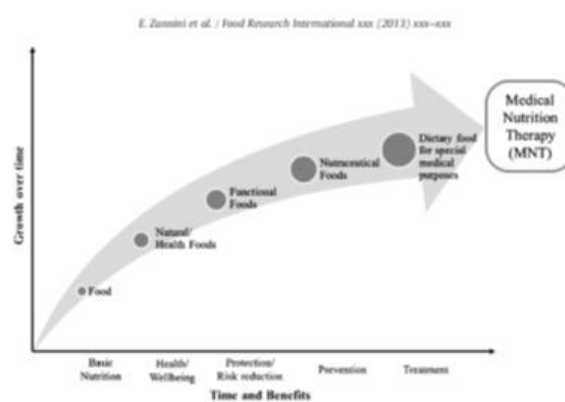
Figure 1.1 Evolution of food and consumer behaviour over time (Zannini et al. 2013).

European products used in the MNT, are called 'dietary food for special medical purposes' and are regulated by the EU directive 1999/21/EC. These foods, are prescribed in the treatment of diseases, disorders or medical conditions, under medical supervision, by counsellors, dieticians and nutritionists.

The term "medical nutrition therapy" (MNT) was introduced in 1994 by the American Dietetic Association to better articulate the nutrition therapy process. It is defined as the use of specific nutrition services to treat an illness, injury, or condition (Pastors et al. 2002, Hager et

al. 2003). Substantial evidence exists that MNT is an effective treatment for many medical conditions (Hager et al. 2003, Harsha et al. 1999, Fujioka 2002, Pastors et al. 2002).

Medical nutrition therapy (MNT) has also proven to be efficient in minimising costs, while improving the patient's quality of life (Allred et al. 1996, Lorig et al. 1999, Carey and Gillespie 1995). The MNT products are basically formulated and/or specially processed foods that are used for the dietary management of patients.

Amongst the medical foods, low-protein/protein-free (LP/PF) foods have been shown to improve the physical manifestation of metabolic and genetic disorders in patients with amino acid or protein-related diseases, such as Phenylketonuria (PKU), chronic kidney disease, celiac disease (Aparicio et al. 2000, Marzocco et al. 2013, Lee and Newman 2003, Sethi et al. 2011) and other illness.

1.2 Proteins intake limit values in LP/PF diets for patients with metabolic and genetic disorders

Dietary treatment involves complex food choices. Labelling standards and guidelines are therefore an important public health measure to help protect the long-term health of individuals who experience an injury or illness associated with the consumption of specific substances present in foods. By providing information to consumers, nutrition labels and health claims on foods, contribute to the achievement of health objectives.

A description of the major diseases and consequences associated with protein consumption and general guidelines and consensus-based tools to adopt in diets with protein restriction, is underlined below, for the main patient categories who may benefit from LP/PF diets.

Phenylketonuria (PKU)

PKU is an inherited metabolic disorder caused by deficiency in the phenylalanine (Phe) hydroxylase enzyme (PAH), which converts Phe to tyrosine. If left untreated from birth, this results in a rapid accumulation of toxic concentrations of Phe in the blood, causing detrimental effects on brain development and function (Ahring et al. 2009).

Nutrition therapy, is still the primary treatment for PAH deficiency (Bowersox, Panel and Panel 2001, Smith et al. 1993). The goals of lifelong nutrition therapy include normal physical growth and neurocognitive development, maintenance of adult health, and normal gestational outcomes in pregnant women with PAH deficiency. Knowledge of metabolism, the pathophysiology of PAH deficiency, and the role of nutrition has led to new and improved

treatment options, modified LP foods, and medical foods that provide protein equivalents and other nutrients when intact protein food sources must be limited (Singh et al. 2014).

Modified LP food are products specially formulated to contain less than 1g of protein per serving. LP modified food products are intended for use under the direction of a physician for the dietary treatment of hereditary metabolic diseases. Examples of commercially available LP food products include breads, pasta, baking mix, and imitation cheese (Singh et al. 2014).

A low-Phe diet currently forms the principal strategy to limit Phe accumulation in the blood and, therefore, in tissues such as the brain (Greve et al. 1994). The low-Phe diet restricts the intake of high-protein foods, and the remaining nutritional requirements must be obtained from Phe-free amino acid supplements (protein substitute) and special or natural foods that are low in Phe. The mainstay of dietary therapy of PKU is to provide a minimum amount of phenylalanine especially to the child patients, which does not increase serum-Phe, but allows a normal growth pattern. The choice of food is limited for phenyl-ketonuric children on a low-Phe diet. Usually, gluten-reduced bakery products are designed to meet requirements of both PKU and celiac patients.

While existing PKU management guidelines typically focus on parameters such as screening, age at initiation of treatment, target Phe concentrations and frequency of Phe monitoring, they do not make recommendations regarding the specifics and practicalities of diet administration, and there is certainly no consensus opinion based on solid scientific rationale. Furthermore, while the concept of using diet to achieve Phe targets is straightforward, there is substantial variation in dietary guidelines, not only among countries, but also among treatment centres within a given country (van Spronsen and Burgard 2008, Blau et al. 2009).

According to guidelines values reported in literature, the treatment goal with dietary restriction is to maintain blood Phe concentrations within defined target limits which may vary from country to country (e.g. 120–360 µmol/L [2–6 mg/dL]) (Giovannini 2007, van Spronsen et al.2008). 'Free foods' (i.e. foods that are so low in Phe that they can be permitted without measurement) comprise both of naturally occurring foods and manufactured low-Phe foods.

Definitions of 'free' natural foods varied widely (Table 1.1), but always included foods such as oils, vinegars, sugars, honey, jam, spices, apple juice, or some types of candies or sweets. The classification of fruits and vegetables varied widely among centres (Table 1.1), from all being allowed without restriction (Spain) to all being counted within the daily Phe allowance (Italy and Turkey). Other centres adopted an intermediate position, usually based on threshold levels of Phe content of fruits and vegetables. For special manufactured low-protein foods, six centres suggested they could be allowed 'freely' if they contained Phe <20–25 mg/100 g.

Country	Definition of 'free' food	Definition of low-Phe 'special' food (mg Phe/100 g)	Phe calculation for fruits and vegetables
B	Foods known to be very low in Phe	Not defined	Up to 20 mg/100 g are 'free'
D	<10 mg Phe/100 g	Not defined	Phe calculated for all initially
DK	<25 mg Phe/100 g	<25	Up to 25 mg Phe/100 g are 'free'
E	Foods known to be very low in Phe	<20	All are 'free'
I	Foods known to be very low in Phe	Not defined	None are 'free'
N	<25 mg Phe/100 g	<25	Up to 25 mg Phe/100 g are 'free'
NL	5 mg Phe/serving	Special low-protein foods allowed freely	Low-Phe fruits/vegetables up to 5 mg Phe per serving are 'free'
PL	<20 mg Phe/100 g	<20	Phe calculated for all initially
TR	Foods known to be very low in Phe	Not defined	Only apple juice is 'free'
UK	<20 mg Phe/100 g	<20	Up to 50 mg Phe/100 g are 'free'

Table 1.1 Use of phenylalanine-free ('Phe-free') foods (Ahring et al. 2009).

Because individuals with PKU are unable to catabolize PHE to TYR, daily intact protein intake must be limited to only the amount that provides the PHE required for anabolism. Recommended ranges for PHE, TYR, and total protein intake by age are reported in Table 1.2 (Singh et al. 2016)

AGE	PHE (mg/day)	TYR (mg/day)	Protein (g/kg/day)
Infants to < 4 years			
0 to < 3 months	130–430	1100–1300	2.5–3.0
3 to < 6 months	135–400	1400–2100	2.0–3.0
6 to < 9 months	145–370	2500–3000	2.0–2.5
9 to < 12 months	135–330	2500–3000	2.0–2.5
1 to < 4 years	200–320	2800–3500	1.5–2.1
After early childhood			
> 4 years to adult	200–1100	4000–6000	120–140% DRI for age
Pregnancy and lactation			
Trimester 1	265–770	6000–7600	≥ 70
Trimester 2	400–1650	6000–7600	≥ 70
Trimester 3	700–2275	6000–7600	≥ 70
Lactation	700–2275	6000–7600	≥ 70

Table 1.2 Guidelines for PHE, TYR, and protein intake for individuals with PKU.

The evidence suggests that individuals who consume "complete" Phe-free medical foods (contributing all necessary nutrients other than Phe) and are adherent to their daily consumption recommendation, are not at risk for nutrient deficiencies or excesses (Singh and MacRitchie 2004)

Chronic kidney disease CKD

CKD or chronic renal failure (CRF) is a term that includes all degrees of decreased renal function, from damaged–at risk through mild, moderate, and severe chronic kidney failure. It can arise from many heterogeneous disease pathways that alter the function and structure of the kidney irreversibly, over months or years (Webster et al., 2017). The diagnosis of CKD is based on establishing a chronic reduction in kidney function and structural kidney damage. The best available indicator of overall kidney function is the glomerular filtration rate (GFR), which equals the total amount of fluid filtered through all of the functioning nephrons per unit of time (Levey et al. 2003).

International guidelines define CKD as decreased kidney function shown by GFR of less than 60 mL/min per $1\cdot73$ m^2, or markers of kidney damage, or both, of at least 3 months duration, regardless of underlying cause (Levey et al. 2011).

Outcomes of CKD include not only kidney failure, but also complications of decreased kidney function and cardiovascular disease (Levey et al. 2003). Diabetes and hypertension are the main causes of CKD (Coresh et al. 2003) .

Patients with CKD are often subjected to diet restrictions and a diet low in protein is sometimes recommended to try to slow the progression of kidney disease. Several studies confirmed that low protein diets can delay the end-stage of kidney disease (Fouque and Laville 2009, Mitch 1991, Mircescu et al. 2007).

Reduced dietary protein intake has been reported for more than a century to improve many uremic symptoms in advanced chronic renal failure (Mitch 1991, Giovannetti and Maggiore 1964). More than two decades ago, it was reported that a hypoproteic diet may also slow down the rate of decline in renal function (Kasiske et al. 1998, Fouque et al. 2000) and postpone the initiation of renal replacement therapy (RRT) (Walser and Hill 1999)

The Recommended Daily Allowance (RDA) represents the intake of nutrient that is safe for 95% of the general population: for proteins, the RDA value is 0.8-1.0 g/Kg/day. Hence LP regimens are featured by a dietary protein intake < 0.8 g/Kg/day (D'Alessandro et al. 2013, Millward and Jackson 2004, Afolabi et al. 2004).

Different dietary protein regimens have been proposed for patients with CKD: a conventional low protein diet (LPD) providing 0.6 g/kg per day, (MacKay, Addis and MacKay 1938) a very LPD (0.3 g/kg per day) supplemented with essential amino acids, or a very LPD (0.3 g/kg per day) supplemented with essential amino acids (EAA) and ketoacids (KA) (Aparicio et al. 2012). However, protein restriction is only a part, though a very relevant one, of a more complex dietary management of CKD patients. Phosphate intake should be reduced (700–400 mg/day) as well as sodium intake (2–3 g/d). Dietary energy intake must cover energy requirement up to 35 Kcal/Kg/d for patients < 65 years and 30 Kcal/Kg/day for patients > 65 years old. Qualitative aspects of foods (plant or animal origin, fresh or preserved, cooking modality etc.) are important as well. The main characteristics of the dietary therapy in CKD are resumed in Table 1.2

Nutrients	Normal diet	LPD	VLPD
Protein, g/Kg/d	0.8	0.6	0.3-0.4
Prevalent origin of proteins	Mixed	Animal	Plant
Phosphate, mg/d	700-800	500-600	300-400
Sodium, g/d	2.3	2.3	2.3
Supplements			
Free-protein products use	Optional	Yes	Yes
EAA + KA	No	Optional	Yes
Calcium, g/d	Optional	0.5-1.0	0.5-1.0
B12 Vitamin	No	Optional	Yes
Iron	No	Optional	Yes

Table 1.2 Dietary composition of low-protein diets for CKD patients (Bellizzi et al. 2016, D'Alessandro et al. 2013).

In summary, protein restriction, including reduction of phosphorus and salt, together with adequacy of energy intake, represent the general characteristics of the nutritional therapy for CKD patients. Moreover, the severity of restrictions and the amount and quality of food and supplements should be defined in accordance with the advancement of CKD and the clinical conditions under which it is observed (Bellizzi et al. 2016).

Celiac disease (CD)

CD also known as gluten-sensitive enteropathy and non-tropical sprue, is an immune-mediated disorder caused by the ingestion of gluten in genetically predisposed individuals (Catassi and Fasano 2008, Briani, Samaroo and Alaedini 2008). CD can occur at any age and is characterized by a broad range of clinical presentations (Tajik 2014). "Typical" and "atypical" symptoms of celiac disease patients are mentioned in Figure 1.2

Typical symptoms	Atypical symptoms
abdominal pain	fatigue
diarrhea	dental enamel defects
chronic	aphthous ulcers (canker sores)
constipation	arthritis and arthralgias (joint pains)
weight loss	low bone mineral density, fractures of bones
vomiting	elevated liver enzymes
Malabsorption	delayed puberty
malnutrition	cerebellar ataxia
iron deficiency anemia	recurring headaches
dermatitis herpetiformis	peripheral neuropathy
short stature	seizures
abdominal distension	psychiatric disorders, including anxiety, panic attacks, depression

Figure 1.2 Typical and atypical symptoms of celiac disease (Tajik 2014).

Besides CD and wheat allergy, there are cases of gluten reactions in which neither allergic nor autoimmune mechanisms are involved. These are generally defined as gluten sensitivity (Anderson et al. 2007). Although symptoms and signs of CD have been recognised for more than 100 years, it was in the 1940s that the Dutch paediatrician Dicke established a link between the protein component of wheat (gluten) exposure and CD (van Berge-Henegouwen and Mulder 1993).

Gluten is the structural protein component of the grains wheat, rye and barley, which are the basis for a variety of flour- and wheat-derived food products consumed throughout the world (Sapone et al. 2011). The principal toxic components of gluten are a family of closely related prolamin and glutamine-rich proteins called gliadins (Shan et al. 2002). However, given the enormous biological diversity and unusual chemistry of gluten proteins, and the absence of satisfactory assays for gluten toxicity, the structural basis for gluten toxicity in CD remains unclear (McAdam and Sollid 2000).

If undiagnosed or untreated, CD can lead to other serious health consequences such as vitamin deficiency, osteoporosis and cancer (Schweizer, Oren and Mearin 2001, Kemppainen et al. 1999).The only treatment for celiac disease is a lifelong gluten-free diet (Catassi and Fasano 2008).

A wide range of GF wheat substitutes are specifically manufactured for patients with CD. Although no gluten consumption is the ideal treatment for celiac disease, it is difficult to avoid even a minimal degree of gluten contamination. The lowest amount of daily gluten that

causes damage to the celiac intestinal mucosa over time (the gluten threshold) is 10 to 50 mg per day (a 25-g slice of bread contains approximately 1.6 g of gluten) (Catassi et al. 2007).

The new Codex Alimentarius (Motarjemi 1991) regulation endorses a maximum gluten contamination of 20 ppm in gluten-free products; this is a safe threshold even for patients who eat large amounts of wheat substitutes. The Food and Drug Administration is still in the process of defining safe gluten thresholds.

In addition to the above described pathologies and for which it is possible to identify general guidelines about proteins intake limit values to be observed in LP diets, a number of other patient disorders and diseases may benefit from a diet with LP/PF foods. These are resumed in Figure 1.3.

Disorder/disease	Type/incidence/ prevalence	Affected enzyme/transporter	Description		
Inborn errors of metabolism (IEM)	3–4:1,000 live births	Over 250 known neurometabolic genetic disorders	Low protein diets and medical therapy to restrict accumulation of toxic metabolites, replace any blocked metsbolites, induce excretion of metabolite build ups, high vitamin doses to reduce enzyme activities, replace nutrients, continuous dietary and progress monitoring, early start		
			Metabolite/process	**Symptoms**	**Dietary treatments**
Phenyl-ketonuria (PKU)	Metabolic 1:10,000 livebirths (Europe), 1:15,000 (USA), 1:50,000–25,000 (Latin America), 1:15,000–100,500 (Asia) to 1:100,000 livebirths (Finland)	Phenylalanine hydroxylase	Phe	Mental retardation, seizures, autism and other neurological disorders, fair hair, eczema, convulsions tremors	Yes, phe-restricted diet very soon after birth, tyr supplements (hydroxylase deficiency)
Tyrosinaemia type I (TTI)	Metabolic 1:200,000 births (France)	Fumaryl-acetoacetate hydrolase; most common error of sulphur metabolism	Phe, tyr	Liver disease, renal tubular dysfunction, risk of liver cancer, peripheral neuropathy, ocular lesions	Yes, low-protein diet with no phe/Tyr, low-protein diet (1–1.5 g/kg/d) and, to sustain exclusion of galactose and fructose
Tyrosinaemia type II (TTII)	<1:250,000	Genetic deficiency of hepatic cytosolic tyrosine aminotransferase – the rate limiting enzyme – tyrosine catabolism	Phe, tyr	Ocular/cutaneous painful palmoplantar hyper-keratotic lesions, ataxia, development delay, tremor, convulsions, mental impairment, nystagmus, recalcitrant bilateral keratitis	Dietary restriction of tyr and phe in infancy to prevent cognitative impairment, low protein diet – huge problem of adherence
Disorder/disease	**Type/incidence/ prevalence**	**Affected enzyme/transporter**	**Metabolite/process**	**Symptoms**	**Dietary treatments**
Homo-cystinuria (HC)	Metabolic 1:40,000–15,500 or 1:20,000–60,000 births (Central Europe or Europe), 1:3000–12,500 (Qatari population) down to 1:6400 (Norway)	Cystathatione β-synthase	Met, homocysteine	Mental retardation, osteoporosis, thromboembolic episodes, ectopia lentis	Yes, particularly in maternal cases where met and cys are supplemented into low-protein diet
Alkaptonuria (AK)	1:100,000–250,000, 1:19000 in Slovakia, also reported as 1:250,000–1,000,000 live births	Homogentisic acid dioxidase	Tyr, phe	Degenerative arthritis of spine and large joints, darkening of urine upon standing, kidney stones, valvular heart disease, prostate stones, also behavioural problems associated with poor dietary compliance	Yes, 1.5 g protein/kg body weight in children. Also reported that ascorbic acid (1–10 g/day) could replace low protein diet, dietary restriction of tyr and phe
Maple syrup urine disease (MSUD)	1:85,000, with 1:176–200 for the Pennsylvania Mennonite population	Mitochondrial branched-chain α-ketoacid dehydrogenase complex	Leu, Ile, val and associated ketoacids	Poor feeding, seizures, coma, neuro-developmental delay, poor feeding, sometimes death, smell of maple syrup in the urine, episodic encephalopathy particularly with seizures	Dietary restriction-branched chain amino acids. Protein/non-essential amino acids limited to 2–3 g/kg/d, 60–90 mg/kg/d leu, 40–50 mg/kg/d ile and val, 250 mg/kg/d glu and ala for asymptomatic neonate. Adult sufferer – 7–9 g/d protein, 90% dependant on medical foods daily
Propionic acidaemia (PA)	1.2:100,000 births, 2:100, 000 neonates,	Deficiency of propionyl-CoA carboxylase	Ile, val, the, met	Neonate illness, acidosis, mental retardation, neurological	Low protein diet, emergency protein-free high-energy diet

			Metabolite/process	Symptoms	Dietary treatments
				problems, infant-failure to thrive, vomiting, retardation, older-intermittent acidosis	during infections. Liver transplant has also been investigated

Disorder/disease	Type/incidence/prevalence	Affected enzyme/transporter	Metabolite/process	Symptoms	Dietary treatments
Methyl-malonic-academia (MMA)	MMA ranges from 1:115,000 in Italy to 1:169,000 in Germany	Methylmalonyl-CoA mutase	Ile, thr, met, val	Poor feeding, psychomotor retardation, muscle tone abnormality, respiratory distress, seizures, loss of consciousness	Yes
Urea cycle disorders (UCD) include other deficiencies; carbamyl phosphate synthetase I, ornithine trans-carbamylase, arginino-succinate synthetase (type 1 or classic citrullinemia), arginino-succinate lyase-N-acetyl glutamate synthetase, arginase, hyperargininemia	1:8200 live births in USA 1:39,000 live births in Finland	Protein degradation pathways	Hyper-ammomaemia, generally dietary restriction of all amino acids to levels required for anabolism	Neonate-often lethal; infancy-vomiting, anorexia, protein intolerance; older-intermittent drowsiness, behavioural problems, neurological abnormalities; chronic mental retardation, neurological symptoms e.g. decreased level of consciousness, abnormal motor function, vomiting	Yes, low protein diet with arginine supplements and sodium benzoate phenylacetate. Emergency protein-free high-energy diet during infections
Aspartylglucos-aminuria (AGA) a type of lysosomal storage disease (LSD)	0.67:100,000 live births globally for AGA and 1:4000–9000 live births globally for LSD	Aspartylglucos-aminidase	Asn	Progressive psychomotor retardation, facial dysmorphism, skeletal abnormalities	Possibly
Familial hyper-lysinemia (FHL)	Unknown, but rare	Saccharopine dehydrogenase lysineketoglutar-ate reductase	Lys	Often no symptoms, in rare cases they have mental retardation	Yes, if diagnosed

Disorder/disease	Type/incidence/prevalence	Affected enzyme/transporter	Metabolite/process	Symptoms	Dietary treatments
Trimethyl-aminuria (inherited type) (TMA)	Estimated at 1:40,000 in white British population, 1:3000 in Jordan, 1:400 in New Guinea	Flavin-containing mono-oxygenase 3	Trimethyla-mine and its precursors — choline, lecithin, carnitine,	Fishy odour from sweat, breath and urine, results in psychological and social symptoms but no physical manifestations	Yes
Nonketotic hyper-glycinemia (NKHG)	Incidence 1:47,455 and prevalence 1:782, 951 — Portugal	Deficiency in glycine cleavage system e.g. P — protein or T — protein activity	Gly	Neonatal form — coma, seizers, colonic jerks, hypotonia, many die from non-neonatal form — seizures, developmental delay, ataxia, spasticity	Sometimes
Propionic acidemia (PA)	Incidence 1:165,000–300,000 globally	Propionyl-CoA carboxylase	Val, ile, thr met (and odd chain fatty-acids)	Early onset form — vomiting, ataxia, poor feeding, tachypnea, academia, hemiplegia, coma; Late onset form — vomiting, psycho-gical and mental disabilities	Yes
Hyperprolinemia type I (HPTI)	Unknown	Proline oxidase	Pro	A symptomatic, some have neurological symptoms	Yes
Hyperprolinemia type II (HPTII)	Unknown	Urine has Δ-1-pyrroline-5-carboxylate dehydrogenase	Pro	Seizures, mental retardation, sometimes asymptomatic	Yes
Cystinuria (CI)	Prevalence 1:7000 worldwide, 1:2500 Jewish Israelis of Libyan origin, 1:100,000 Sweden	Cystine and diabasic amino acid transport (type 1 encoded by SLC3A1 gene; non-type 1 encoded by SLC7A9 gene)	Cys, met metabolites, ornithine, arg, lys	Cystine stones in kidneys, and/or bladder and/or ureter, pain	Yes

Disorder/disease	Type/incidence/prevalence	Affected enzyme/transporter	Metabolite/process	Symptoms	Dietary treatments
Dicarboxylic aminoaciduria (DCAA)	Incidence 1:35,000	Glutamate transporter SLC1A1	Glu, asp	High urine level of aspartic acid and glutamic acid; rarely mental retardation	Possibly
Glutaric acidemia type I (GATI)	Overall prevalence 1:100,000	Glutaryl-CoA dehydrogenase	Lys, hydro-xylysine, trp	Dystonia, macrocephaly, neurological symptoms	Sometimes patients with a mild form, always in other forms protein restricted to 2–2.5 g/kg/d. Young patients need a low-lysine moderate-tryptophan diet. Pro-tein stopped if unwell and carbohydrates increased
Hydroxymethyl-glutaric aciduria (HMGA)	Unknown	3-hydroxy-3-methylglutaryl-CoA lyase deficiency	Car	Severe hypoglycaemia, short-lasting lethargy	Lifelong with low fat and protein restriction, rich in carbohydrates
Lysinuric protein intolerance (LPI)/cationic aminoaciduria	1:60,000 in Finland, 1:57,000 in Japan (1:119 are carriers), 140 individuals worldwide	SLC7A7: Y + L amino acid transporter 1 enzyme is mutated at the genetic level	Lys, arg, orn below normal. Ser, gln, cit, pro, ala, glu are increased	Infant: vomiting, diarrhoea, episodes of stupor and coma after a protein-rich meal, failure to thrive, poor feeding,	Long term dietary protein restriction combined with citrulline and nitrogen scavenging drugs, with many

Disorder/disease	Type/incidence/prevalence	Affected enzyme/transporter	Metabolite/process	Symptoms	Dietary treatments
				hepato-splenomegaly, muscular hypotonia; older: poor growth, osteoporosis, lung and kidney effects, haemato-logical abnormalities, acute pancreatitis	patients deficient in energy, essential amino acids, vitamins and minerals
Aspartyl-glucosaminuria (AGU)	1:18,000	Inadequate aspartyl-glucosaminidase activity (AGA)			
Chronic kidney disease (CKD, which includes KI and CRI below)	Acquired 7.2% (>30 years), 23.4–35.8% (>64 years) based on MDRD[a], up to 45.6 and 71.0% (males > 75 years and > 85 years, respectively, USA) and 10.25% in Chinese populations	n/a	n/a	n/a	Frequently, low-protein diet (~0.6 g/kg body weight/d), combined with drug treatment
Kidney insufficiency (KI)/chronic renal insufficiency (CRI)	Generally acquired 9.46, 5.18, 3.11 and 1.38% in American whites, African Americans, Hispanics and Chinese	n/a	n/a	n/a	Sometimes
Coeliac disease (CD)	Toddlers — 1:77 Sweden, school children — 1: 67–99 Finland, 1:230 Italy, population 2–13:100,000	Metabolic or acquired aspects to the disease	n/a	n/a	Yes (absence of gluten and related peptides is necessary)

Figure 1.3 (Zannini et al., 2013)

1.3 LP/PF cereal-based foods

"Protein-free" food is an idea that was "Made in Italy" and developed over a period when the availability of dialysis was very limited (Giovannetti 1986) . The systematic use of protein-free food as a medical treatment for kidney diseases is limited to Italy, where these products are available, free of charge, for CKD patients and where up to half of the protein intake derives from bread and pasta (12-14 g of protein per 100 g).

Replacing these foods, with their LP/PF counterparts, allows patients to reach the target protein intake without making any substantial changes in dietary habits (Piccoli et al. 2016). Nowadays, these products are increasingly in use also in other countries around the world.

The PF/LP food products represent a very important tool for the implementation of a low-protein diet in CKD treatment, to ensure adequate energy supply, reducing the production of nitrogenous waste products (D'Alessandro et al. 2013).

LP/PF bakery products are also designed to meet requirements of both PKU and celiac patients (Özboy 2002). These products consist mainly of carbohydrates (starch) and are virtually free of nitrogen, potassium and phosphorous as well as also being low in salt content.

They effectively replace analogous staple foods (bread, pasta, biscuits) making it possible to reduce the intake of protein of low biological value and thereby allowing the adequate intake of animal proteins whilst ensuring a high energy intake (D'Alessandro et al. 2013).

LP/PF diets are high in carbohydrates, which are contraindicated for patients with nephrotic syndrome or diabetes mellitus (Bellizzi et al. 2016).

The growing demand for high-quality PF baked products, represents a challenging task in terms of technology and nutrition due to low quality especially for baking of GF flour and a lack of fibres, vitamins and nutrients (Morais et al. 2014). In the early years, PF products were far less palatable than corresponding foods, thus limiting patient adherence. In the last few years, however, these foods were further developed and their palatability much improved. It is in fact notable that the risk of "malnutrition" associated with a LP/PF, diet is usually the result of an insufficient energy intake rather than of a low protein supply (Bellizzi et al. 2016).

From a nutritional point of view, most of the cereal-based LP/PF foods currently marketed are a blend of refined or chemically-based food ingredients with unpalatable, frequently artificial flavours, having excessive sweetness to mask the chemical tasting ingredients (drug-like approach). However, a food-like approach to developing medical foods, is a complex process. This is specifically true when the technological aspects of LP/PF foods and, in particular, PF cereal foods are considered (Zannini et al. 2013)

1.4 Gluten-free bread

Bread is a dietary staple for a large percentage of the world's population. It is one of the most consumed products among baked foods (Bakke and Vickers 2007). Its use dates back to the early Egyptian era. Although a variety of grains have been used, wheat flour is most common in bread baking. Wheat flour proteins have long been known to be crucial in relation to bread-making quality, both protein quantity and quality being important (Schofield 1994, Khaktar et al., 2002, Goesaert et al., 2005). Wheat contains two proteins, glutenin and gliadin, which develop into gluten during the mixing and kneading processes.

Gluten provides some unique functional properties in leavened breads. Studied extensively, but not fully understood, gluten is responsible for the protein-starch interaction that provides specific viscoelastic properties in bread dough. These properties are largely responsible for gas cell formation, including stabilization and retention of the gas cells during the proofing and baking process.

A variety of breads have been created as replacements for traditional wheat breads in an attempt to provide alternative LP or GF cereal-based foods; however there are a number of problems associated with the production of these products and, although many gluten-free products are nowadays available in the market, the scientific literature is still lacking on the systematic development of gluten-free breads with properties similar to those of conventional

wheat counterparts. The literature referred to gluten-free bread is limited probably due to the commercial secrecy of the baking industries.

Despite the great progress made in recent years, GF products present on the market are still characterized by low nutritional values and unsatisfactory sensory quality, particularly when compared to their wheat equivalents.

In GF bread production, the absence of gluten, that plays a central role in giving a peculiar texture to baked goods, making them soft and aerated (Dobraszczyk et al., 2001), makes the whole process problematic penalizing also the sensory quality of the final products.

1.5 GF-bread ingredients and new trends

Elimination of gluten in celiac diets, increases the role of starch in providing structure and texture to GF formulations. From a nutritional point of view, most of the GF products are therefore basically "starchy" foods. The most important starch sources in such products are corn, wheat, tapioca, rice, cassava, and potato (Lazaridou et al., 2007; Mahmoud et al., 2013).

Other widely used ingredients in GF bread formulations include sorghum (Schober et al., 2005; Onyango et al., 2009, Marston et al., 2016), legume flours (soya, chickpea, pea) and pseudo cereals (Aguilar et al., 2015). The origin and type of starch influences microstructure, dough rheology, water retention, final structure and quality of the final product (Witczak et al., 2016).

The high amount of starch from different origin in GF formulations makes the product more sensible to staling and reduces its shelf-life (Ahlborn et al., 2005, Ziobro et al., 2012). Also, such a high content of carbohydrates makes these type of products, unsuitable for people who suffer from diabetes.

In GF formulation for baked goods, the addition of hydrocolloids to simulate the viscoelastic behaviour of the wheat dough, is quite common, because these ingredients have a strategic role in making dough workable and in improving the texture of the final product (Gallagher et al., 2004; Lazaridou et al., 2007, Masure et al., 2016). Hydrocolloids or gums comprise of a number of water-soluble polysaccharides with varied chemical structures providing a range of functional properties that make them suitable for different applications in the food industry (Rosell et al. 2007). Besides being applied as gluten substitutes in GF breads, hydrocolloids have been used in foods to improve texture, to slow down the starch retrodegradation, to increase moisture retention, and to extend the overall quality of the product during time (Rojas et al. 1999).

The impact of different hydrocolloids on the characteristics of dough and bread quality is known to be highly dependent on raw materials, the nature and quantity of hydrocolloids.

Several hydrocolloids have been applied in GF breads such us Xantham gum (Kulp et al. 1974; Moore et al. 2004; Ahlborn et al. 2005; Schober et al. 2005; Moore et al.2006; Lazaridou et al. 2007, Hager et al., 2013) HPMC (Nishita et al. 1976; Kang et al. 1997; Cato et al. 2001; Sivaramakrishnan et al. 2004; McCarthy et al. 2005; Lee and Lee 2006, Van Bockstaele et al., 2016), CMC (Cato et al. 2002; Lazaridou et al. 2007) , carrageenan (Kang et al. 1997;), locust bean gum (Acs et al. 1997; Kang et al. 1997), pectins (Gambus et al. 2001; Korus et al. 2006; Lazaridou et al. 2007), agarose and β-glucan (Lazaridou et al. 2007), konjac gum (Moore et al. 2004), gelatin (Ribotta et al. 2004). Among all, xanthan gum and HPMC appear to be the best in mimicking the gluten properties, and therefore are the most used (Anton and Arfield, 2008).

Commercial mixtures for GF dough contain mostly carbohydrates, which have a negative influence on the quantity of proteins present in the diet of celiacs, because bakery products are an important source of aminoacids in a diet of healthy people (Matos Segura and Rosell 2011).

Addition of non-gluten proteins, in the production of GF bread, is especially interesting, as those substances have both nutritional and technological role. Their addition reduces amino acid deficits, and impacts on structure and texture forming properties of the dough, as well as the colour and sensory properties of the final product, in this way affecting its consumer acceptance.

Proteins from different botanical sources are therefore widely used in the enrichment of GF bread formulations with the role of nutrients and as structure and texture forming agents. Among cereal proteins, zein and kaffirin have been applied for gluten-free bread supplementation (Deora et al. 2015; Schober et al. 2011). Andersson et al. (2011) observed that the addition of corn protein in the presence of hydrocolloids positively influences dough rheology, improves bread structure and increase its volume. The addition of dairy proteins strengthens structure, improves texture and color of bakery products and slows down its ageing (Ziobro et al. 2016).

The use of proteins in GF bread formulation affects dough rheology and bread characteristics such as crumb density, crumb hardness and specific volume (Horstmann et al. 2017). Egg proteins are also used as a functional ingredient in GF bread (Deora et al. 2015). The high functionality of this ingredient is related to its foam stabilizing properties, which are important for gas retention, and structure stabilization of the crumb (Schoenlechner et al. 2010; Tsatsaragkou et al. 2014).

Another group of proteins applicable in GF products is derived from legume seeds such us soybean. They are nutritionally valuable due to high lysine content, which is a limiting amino acid in cereal products (Marco and Rosell 2008). Soya protein, also high in its essential amino

acid lysine content, can be added in the form of high-protein soya flour or as a soya protein isolate and can lead to an increase in crumb texture and bread volume (Houben et al., 2012).

Other proteins tested in the production of GF include collagen and lupine proteins (Ziobro et al. 2013). Furthermore, hemp flour and hemp protein concentrate have recently become widely used for the production in GF baked gods, as natural nutritional and structure-forming agents in GF starch bread. Korus et al. (2017) observed that supplementation of starch bread with preparations based on hemp, limited crumb hardening and amylopectin recrystallization during storage.

Improved dough texture and appearance have also been reported when using enzymes in bread making. In GF and also in gluten-contending recipes, enzymes are very often added to improve the dough-handling properties and to increase the final baking quality. Depending on the enzyme activity, the water-binding capacity, the shelf-life, the retrogradation and the crumb softness can be influenced positively (Rossell, 2009). Some of those enzymes often used in GF bread production are the starch-modifying amylase, cyclodextrin glycosyltransferases or the protein-connecting transglutaminase (TGase). In addition to this, glucose oxidase, laccase and proteases can be found in the recipes (Moore et al., 2006; Goesaert et al., 2008). Some of these enzymes are essential for reaching higher quality in GF bread. Gujral et al. (2003) successfully used α-amylase of intermediate thermostability and cyclodextrin glycosyl transferase in rice bread formulation. Diez Poza (2002) studied the activities of transglutaminase as a new tool in the manufacture of baked good and Moore et al. (2006) evaluated the impact of transglutaminase at different levels in conjunction with different protein sources such as soya, skim milk or egg powder. This enzyme acts by modifying proteins of different origin by amine incorporation, cross-linking or deamidation. The authors concluded that transglutaminase can be successfully applied to GF flours to improve their breadmaking potential by promoting network formation

Recent studies have highlighted the need for an improvement in the nutritional quality of cereal based GF products. According to the literature, celiac individuals have generally a low intake of fibres due to their GF diet (Thompson 2000). From this perspective, the incorporation of dietary fiber in GF breads appears to be a promising approach. The possibility of enriching GF bread with dietary fibre have been investigated (Gallagher et al., 2004).

The addition of dietary fibres, can help in improving the nutritional quality of GF breads, but also in ameliorating texture, sensory characteristics and shelf-life of foods, thanks to its water binding capacity, gel forming ability, textural and thickening effect. Among the hydrocolloids, cellulose and its modified forms serve also as dietary fibre (Mudgil and Barak, 2013). Even though some research has been carried out recently in relation to the ways in which

dietary fibres can be used in GF formulations (Hager et al., 2011; Sabanis et al., 2009), the limited amount of available data does not provide a substantial amount of support when attempting to explain the consequences of combining fibres to enhance the appearance, taste and smell of GF bread. Several GF grains, such as the pseudocereals are characterized by an excellent nutrient profile. Thus, an increasing trend in research is focusing on their use in the formulation of high quality, healthy GF products.

Developing GF breads from wholegrain and pseudocereal flours like buckwheat, amaranth, flax, quinoa, brown rice, teff, legumes, sorghum and nuts would be an ideal option for improving the nutritional quality and the dietary fibre content of GF breads (Niewinski 2008). Studies have shown that, dietary fibre content is significantly higher in buckwheat seeds in comparison with amaranth and quinoa, which have fibre levels comparable to those found in common cereals (Alvarez-Jubete et al., 2009; Moore et al. 2004). The incorporation of buckwheat flour in a GF formulation produced good quality bread. Bread had low specific volume and high crumb hardness, but the staling rate was lower than in wheat bread.

Apart from native starch, GF products may contain starch modified by chemical, physical or enzymatic treatment. Their main role is usually an improvement of texture properties and staling prevention. Resistant starch (RS) preparations improve the nutritional quality of GF food. RS also represents a font of fibres and an alternative to the most commonly used starches in GF formulations. It is classified as a dietary fibre because it is not digested in the small intestine (Birt et al., 2013) and it can also help in preventing some serious diseases.

The studies on the influence of resistant starch on quality of starch-based products were also performed on GF food. Korus et al. (2009) analysed the effects of replacement of corn/potato starch in a formulation for GF bread with preparations of corn and tapioca RS. The addition of RS caused an increase in the total amount of dietary fibre. It was also observed that the preparations had significant influence on dough rheology. Bread supplemented with RS was characterized by softer crumb in comparison to control. Other results (Witczak et al., 2012; Ziobro et al., 2012) also indicate that the addition of chemically modified starch in the amounts up to 15% significantly modifies dough rheology, improves bread volume and decreases crumb hardness, improving its elasticity. No positive effects were however observed after the addition of high amylose corn starch. Tsatsaragkou et al. (2014) supplemented GF bread based on rice flour with RS preparation, and found that the presence of RS has little effect on crumb firmness, but significantly improves its elasticity, especially at 15% addition of RS.

The combined use of naturally GF ingredients mentioned above, produced many interesting results related to sensory properties, summarized below. Psyllium husks and HPMC proved to be an effective substitute for wheat gluten in bread-making, as these two materials

stabilise gas cells over complementary ranges of temperature (Haque & Morris, 1994). Dairy powders with high protein contents (80-90 %) produced wheat starch based GF breads with good crust and crumb characteristics, and improved nutritional content (Gallagher et al., 2003). Good-quality GF breads could be formulated from a mix of rice, cassava and active soybean flours (Ribotta et al., 2004). Rice flour is low in prolamins (2.5–3.5 %), and various hydrocolloids can be incorporated to produce bread from a batter of rice flour. HPMC has been found to be the most suitable hydrocolloid to produce rice bread (Gujral & Rosell, 2004). The applications of rice flour can be broadened by covalently cross-linking the rice proteins, either intramolecularly or intermolecularly, to produce a stable network. This is achieved by using microbial transglutaminase and glucose oxidase in combination with 2 % HPMC (Gujral & Rosell, 2004). Hydrocolloids improve the rheological behaviour of the doughs, have pronounced effect on viscoelastic properties and resistance to deformation, and strengthen doughs (Lazaridou et al., 2007; Rosell, Rojas, & De Barber, 2001).

GF breads, developed in the studies mentioned above, had some similarities to conventional wheat flour bread, but there were some differences in colour, taste and texture as well. GF bread developed by using rice flour, hydrophilic psyllium husk and HPMC by Haque & Morris (1994) was whiter than wheat bread, but had a characteristic rice taste. The GF bread developed by Gallagher, McCarthy, Gormle, & Arend (2004) using wheat starch, GF flour, milk powder, and milk proteins had, among other problems, a low specific volume, and an excessively dark crust due to Maillard reaction. Breads made with inulin or fish proteins exhibited similar (excess browning with inulin) or different (rapid staling) defects. The addition of cross linking enzymes and HPMC yielded acceptable rice bread as reported by Gujral & Rosell (2004), but failed to produce acceptable non-sticky dough for the industrial production.

1.6 Rheological Analysis on GF bread doughs

Rheology is the science that studies the flow and deformation of matter and it is widely used in the analysis of complex food systems such as bread doughs, emulsions and so on. In fact, rheology is used to study the properties evolution of bread change between the mixing process (dough) and the final product and can be monitored and studied trough rheological techniques. Many factors affect dough rheology during the time after mixing including relaxation of the stresses induced during mixing, continuing hydration of flour components, and redistribution of water (Khatkar, Bell, & Schofield, 1995).

Dough properties are measured using numerous rheological techniques. The most often used instruments are the farinograph, mixograph, extensograph and alveograph (Mancebo et

al., 2015). The rheological characterisation of GF doughs is usually related to quality indicators of end-products and provides important information for food technologists, allowing the appropriate selection of ingredients to optimise the final product (Lazaridou & Biliaderis, 2009). The mechanical properties of GF doughs are also measured by fundamental rheological methods including oscillatory tests, such as strain and frequency sweeps, as well as creep-recovery tests (Lazaridou & Biliaderis, 2009; Moreira et al., 2011).

Several authors have studied the rheological properties: elastic modulus (G'), storage modulus (G''), and tan δ (G'/G'') of good and poor bread making flours. The rheological response of any material is expressed physically by stresses, strain and strain rate (Petrofsky & Hoseney, 1995). Particularly, dynamic oscillatory testing measures the elastic and viscous component of a material to assess the frequency-dependent properties of material that may provide important parameters of the behaviour of food processing at a large scale.

The design of GF bread-like products therefore involves the study of GF dough rheology and the resulting baked product characteristics, but very little information has been obtained connecting dough and baked product properties.

Keetels et al. (1996) studied the role of starch on the mechanical properties of starch bread and the changes during storage. Preparation of GF bread used xanthan instead of gluten to confer a crumb structure similar to that of bread made from normal wheat flour. The mechanical properties of breads made of potato or wheat starch were measured. The initial modulus and the critical stress for structural collapse increased, and the critical strain decreased. Moreover, the resistance of bread crumb to collapse structure in compression decreased. The mechanical properties of potato starch bread changed more rapidly than those of wheat starch bread.

Lazaridou et al., 2007 studied the effect of hydrocolloids on dough rheology and bread quality parameters in gluten-free formulations based on rice flour, corn starch, and sodium caseinate; the hydrocolloids added at 1% and 2% w/w (rice flour basis) were pectin, carboxy -methylcellulose (CMC), agarose, xanthan and oat β-glucan. The study on the rheological behaviour of the doughs containing hydrocolloids, performed by farinography and rheometry, showed that xanthan had the most pronounced effect on viscoelastic properties yielding strengthened doughs; addition of xanthan to the GF formulation resulted in a farinograph curve typical of wheat flour doughs.

Schober et al., (2007) conducted a study with the aim of improving the quality of GF sorghum bread. The addition of 2% HPMC improved bread based on 105% water, 70% sorghum flour, and 30% potato starch. In temperature ramp tests, sourdough fermentation caused a significantly higher resistance to deformation ($|G^*|$) after gelatinization of the above

batter relative to batters without sourdough. Results suggest that a strong starch gel, without interference of aggregated protein, is desirable for this type of bread.

Fundamental rheological tests, Texture Profile Analysis and standard baking tests were also performed by Renzetti et al., 2008 to study the effect of TGase on GF bread batters, obtained using flours from six different GF cereals (brown rice, buckwheat, corn, oat, sorghum and teff). Fundamental rheological tests showed a significant increase in the pseudoplastic behaviour of buckwheat and brown rice batters when 10U of TGase were used. Overall, the results of this study show that TGase can be successfully applied to GF flours to improve their breadmaking potentials by promoting network formation. However, the protein source is a key element determining the impact of the enzyme.

Rheological properties of GF bread doughs, obtained with different levels of corn starch, amaranth flour (to enhance the nutritional benefits), pea isolate (to increase the protein content), and psyllium flour (as thickening agent and fibre source) were analysed by Mariotti et al. (2009), in order to understand the influence of the different ingredients on the mechanical behaviour of the materials. Results showed that psyllium fibre generally enhanced the physical properties of the doughs, forming a film-like structure, and it presented good bread technological and nutritional quality. The GF bread mixtures that were starch-based could give rise to a quick bread staling, because the lack of an embedding structure allows starch to rapidly swell and gelatinize. The addition of protein and fibre sources improved the experimental GF doughs from a rheological and nutritional point of view, and delayed the staling.

Sabanis and Tzia (2009) conducted a study on different cereal fibres (wheat, maize, oat and barley) added at different level (3, 6 and 9 g/100 g) into a GF bread formulation based on corn starch, rice flour and HPMC. The Doughs' rheological behaviour was evaluated studying the consistency, viscosity and thermal properties. Results showed that maize and oat fibre can be added to GF bread with a positive impact on bread nutritional and sensory properties. The study also showed the potential of developing fibre-rich gluten-free breads in order to increase the dietary fibre intake.

GF bread formulations based on chestnut and rice were studied by Demirkesen et al. (2010). The influence of hydrocolloid blend (xanthan–locust bean gum (LBG), xanthan–guar gum blend) and emulsifier DATEM on the rheological properties of dough formulations were also investigated. Results have shown that all the dough formulations exhibited a shear-thinning behaviour with different power law constants (The power-law index (n) of dough samples at 25 °C ranged from 0.52 to 0.87, the consistency index (K) of the samples ranged from 3.6 to 79 Pa·s^n and the yield stress of the samples ranged from 4.8 to 85.9 Pa). Adding emulsifiers in addition to gums was necessary to obtain the desired physical properties in dough formulations.

Flow and oscillation measurements, both indicated that DATEM had a more pronounced impact on rheological properties of dough, but the authors suggested that it needs to be used with different gums to obtain desired rheological properties and acceptable quality values in GF rice breads. Rice - based GF bread doughs were also characterised through rheological tests by

Matos and Rosell (2013), using a Mixolab® instrument that measures the consistency of a dough subjected to the dual constraints of mixing and increasing temperatures. Differences found in the rheological dough properties from Mixolab® were mainly associated with the presence/absence of protein and starch sources in the dough. Several relationships were found among the rheological properties of dough/batters, the instrumental quality parameters and sensory characteristics of the bread-like products. In general, the highest correlation coefficients (r>0.70) were obtained between the dough's rheological properties and the instrumental quality parameters of the fresh baked products.

Juszczak et al. (2012) studied the effect of inulin on GF dough characteristics, monitoring the influence of inulins with varying degree of polymerization on rheological properties of gluten-free starch-based dough. The share of inulin reduced the values of consistency coefficient as well as storage and loss moduli, and increased creep compliance. Inulin preparation with the highest average degree of polymerization had the strongest impact on viscoelastic properties of the obtained dough.

Moreira et al. (2013) investigated the effect of hydrocolloids on the rheology of GF doughs based on chestnut and chia flour. The simultaneous presence of chia and hydrocolloids modified significantly the rheological properties of doughs. Apparent viscosity at constant shear rate, storage and loss moduli at constant angular frequency decreased with increasing hydrocolloid content, except that the loss modulus of the dough containing tragacanth gum exhibited a reverse trend. Creep-recovery data showed that doughs' elasticity improved with the presence of guar gum (65.9%), HPMC (64.8%) or tragacanth gum (45.8%) at 1.0, 2.0 and 1.0 (g/100 g), respectively.

Burešová et al., (2014) studied the rheological characteristics of GF doughs and their effect on the quality of leavened bread made from: amaranth, chickpea, corn, millet, quinoa and rice flour. The rheological characteristics (resistance to extension R, extensibility E, R/E modulus, extension area and stress at the moment of dough rupture) were obtained performing uniaxial dough deformation. The dough exhibiting stronger resistance to extension, greater extensibility and higher stress at the moment of sample rupture had better bread baking quality.

Burešová et al. (2016) also studied the effect of calcium and sodium caseinate supplements (2 g/100 g) on the behaviour of rice buckwheat dough and comparing it to the effect of xanthan gum and CMC. The addition of caseinates significantly increased dough

weakening, which became similar to dough with xanthan gum. During heating, the gelatinization rate became similar to dough with CMC. Peak viscosity was decreased by the addition of hydrocolloids.

Witczak et al. (2016) studied the rheology and physical characteristics of GF bread dough supplemented with different amounts of potato protein. Linear viscoelastic behaviour was evaluated and creep and recovery tests were also performed. Potato protein at 2–10% level significantly modified rheology of GF dough. According to previous authors (Ronda, Villanueva, and Collar, 2014), results confirmed that proteins from vegetable sources led to more structured dough matrices, higher viscoelastic moduli and steady viscosities, and lower tanδ, instantaneous and retarded elastic compliances; the effect was even more evident with higher protein concentration.

Yazar et al., (2017) performed Large Amplitude Oscillatory Shear (LAOS) flow experiments and baking tests on rice, buckwheat, quinoa, and soy flour doughs to understand if there is any correlation between the non-linear rheological properties and loaf volume. The distributions of elastic and viscous LAOS parameters showed that soy dough has the closest rheological performance to wheat dough among other dough samples, which has the highest protein content.

Martinez and Gomez (2017) conducted a rheological and microstructural study of the most common gluten-free flours and starches during bread fermentation and baking. Maize flour, rice flour, maize starch, wheat starch and potato starch were used in the formulations. Linear viscoelastic properties were studied by small amplitude oscillatory test (SAOS). Creep tests were also performed. Results showed that flour-based doughs exhibited a higher viscoelastic modulus than the starch doughs, indicating a higher consistency of doughs made with flours. This phenomenon has been attributed to the larger particle size and the protein adhesion in flours. In creep tests, among starches, wheat displayed higher compliance values than maize starch at the three fermentation times, which is in agreement with the low consistency (low viscoelastic moduli) of wheat starch doughs. The study showed that changes produced during the fermentation and baking of GF breads depended on the structure and morphology of starch granules and flour particles. In general, results showed that the large and compact flour particles partially maintained their integrity during the kneading process causing doughs to be more consistent and resistant to shear stress, also granular morphology, size, water absorption capacity and pasting temperature affected the way the starches interacted.

1.7 GF-bread systems investigated by thermal analysis

As described in the previous paragraphs, GF formulations are often supplemented with proteins and gums to improve their quality. Non-gluten proteins are typically added to GF applications to improve perceived quality by enhancing Maillard browning and flavour, to improve structure with gelation and cross-linking, and they are also used as foaming agents (Crockett et al. 2011; Gallagher et al. 2004a).

Non-starch hydrocolloids improve dough cohesiveness by inducing attraction between the starch granules and possibly bind them, thereby decreasing their mobility. Furthermore, these agents, temporarily bind water required to gelatinize starch, structure the crumb, increase loaf volume and decrease crumb firmness (Gallagher et al. 2004a).

Water occupies intermediate sites between solvated molecules in food, and thanks to its high mobility, acts as a plasticizer of the whole system (Matveev et al., 2000) for these reasons, water is responsible for many physical properties of food systems. Changes in water distribution may affect the observed textural characteristics of the baked products. Thermodynamically, water in the dough is partitioned into two states when differential scanning calorimetry is used: mechanically and chemically entrapped water, ''unfreezable'' (UFW) and easily removed water, ''freezable water'' (FW) (Vittadini and Vodovotz 2003). FW is the chemically and physically available water fraction involved in reactions occurring within the dough matrix, and behaves like pure water at freezing temperature. This mobile water plays a role in the mechanism of formation of the bread crumb structure, which depends on the number and kind of cross-links formed between the nearest-neighbouring biopolymer chains (Schiraldi et al., 1996). Water also is a key factor for the microbial growth, which can produce degradation processes and texture changes. (Fessas and Schiraldi 2001). The nature of links involving water molecules in food systems is always of hydrogen bonds. Water states and displacements can be investigated with thermogravimetry (TG).

The thermogravimetric analysis, that measures the rate of change in sample mass due to heat transfer (Xu et al., 2005) is a useful tool in distinguishing the main water fraction in carbohydrate systems, suggesting the interaction of different dough components with water (Crockett et al. 2011). Thermal analysis techniques are particularly suitable for analysis of food systems because they are often subject to heating or cooling during processing and have been widely used by many authors in the study of the physical properties of doughs (Walstra, 2002). Depending on the specific link between water and substrate, the strength of the hydrogen bonds within the dough can vary in a narrow range implying differences of ΔH_{vap} (Schiraldi and Fessas, 2003). Unfortunately, changes in the ΔH_{vap} values, in such a complex system, cannot be easily and reliably detected with a standard TG-DSC instrument. However, different states

of water can be recognised because of the different temperatures at which water is released during experimental runs (Schiraldi and Fessas, 2003). DTG curves obtained from food systems indeed show multiple and/or shouldered peaks that can be split into separate contributions through a mathematical deconvolution of the whole trace (Schiraldi et al., 1996; Fessas and Schiraldi, 2001). This allows evaluation of the mass fraction of each state of water within the system. The same treatment can be applied to the concomitant DSC trace to draw the enthalpy change relevant to each DTG peak (Schiraldi and Fessas, 2003). This energy, involved in the water loss in the dough at different stages of the heating process, is affected by the water distribution in the sample that changes with its composition, and can be therefore influenced by the structure formed by the biopolymers in the system. From previous work about wheat dough, it has been observed that the water portion released at temperature below 100°C is attributed to a simply diffusion of the imbibed water (He and Hoseney, 1991). On the other hand, the water fraction released at temperature above 100°C is associated with the gluten network and it is a major determinant to the physical properties of the dough and the final structure (Schiraldi and Fessas, 2003).

The interaction between various dough components and water, starch gelatinization temperatures, water distribution, staling rate etc., for wheat bread dough, has been widely studied trough thermal and thermogravimetric analysis; however, very little literature exists about GF bread dough. Thermal analysis techniques were used in GF formulation, both on fresh doughs/batters and on cooked products. Information about wheat doughs, however, represent an important starting point for studying GF doughs.

As far as wheat flour dough is concerned, Schiraldi et al. (1996) studied the structure and properties of bread dough and crumb, performing a characterization of bakery products through physical and physicochemical investigations. The effects of hydrocolloids (guar and locust bean gums), soluble pentosanes, and whey proteins on staling of bread crumb were investigated using the DSC. The study confirmed the starting hypothesis that the added hydrocolloids would behave as "water binders" and some of them as anti-staling agents, although this action might be indirect.

Starch gelatinization in wheat flour dough of various moisture contents was quantitatively evaluated through thermal analysis techniques by Fessas and Schiraldi (2000). An investigation on simulated baking processes was performed. DSC runs were carried out at constant moisture level (sealed pan) and allowing the water to evaporate from the sample (unsealed pans) to simulate two extreme conditions of a real baking process. One referred to the baking process of the most internal area of the dough and the other to the heating of the most superficial layer respectively. The study concluded that after defining the optimal dough

recipe, it is possible to design the baking progress evaluating the starch gelatinization degree in a simulated baking process with DSC runs. Therefore, the results obtained in the two extreme conditions, using a small amount of sample, cannot represent the whole bread, but only a specific region where the moisture level undergoes well known changes in the course of the baking process. In any real situation, the actual progress of starch gelatinization would be at an intermediate position between the two extremes.

Fessas and Schiraldi (2001) also studied the water properties in wheat flour dough using a thermogravimetry approach. The behaviour of water within a wheat flour dough was investigated preforming thermogravimetric analysis. In wheat flour dough, water is partitioned between coexisting phases which are far from the true thermodynamic equilibrium. The study showed that water is released in two main steps, the first corresponding to a pure diffusion process, the second related to the desorption of water more tightly bound to the gluten network. It was observed that the overall dough moisture, the extent of mixing, the dough resting time after mixing can modify water distribution between phases and the way water is released during heating. The same effect was also recognized in dough samples to which original water-soluble proteins had been added.

Fessas et al. (2008) used a thermal analysis approach to study wheat bread, enriched with buckwheat. The aim of the study was to understand the role of the main components of buckwheat flour (polysaccharides and proteins) in bread making and to assess guidelines for novel recipes for bread from wheat and buckwheat flour blends with improved nutritional properties. Buckwheat contains proteins with poor network-forming abilities, and this limits its use in bread-making. Results of the study showed that the network-forming abilities of buckwheat flour could be improved introducing a de-hulling step in the buckwheat milling process, and adding some buckwheat polysaccharide fractions, isolated from the buckwheat husk. This could have a positive effect on the formation of the crumb structure allowing to obtain bread from de-hulled buckwheat/wheat flour blends with alveolar distribution closer to that of the wheat bread. Results indicated that de-hulling removed some polysaccharide fractions that are relevant to the formation of the crumb structure, thanks to their effect on the phase separation driven by the thermodynamic incompatibility with gluten proteins.

The same analysis techniques used for wheat flour bread doughs, have also been used to study gluten-free doughs. The literature on the study of gluten-free bread using the thermal and thermogravimetric approach is reported below. Many authors studied the staling process. The firming of the crumb is caused by starch crystallization and moisture transfer from the bread crumb. Among the components of bread dough, gluten forms a viscoelastic network that is responsible for slowing down the movement of water (Demirkesen et al. 2014). Therefore,

GF breads, which lack in gluten, have low volume, poor texture and flavour, and stale faster. Other studies are about improving textural properties of the products. The two main problems of GF bread are in fact related to the shelf-life and the final quality of the baked product.

Nunes et al. (2009) studied the impact of emulsifiers on the quality of gluten-free breads and batters. The emulsifiers, added a three different level, were lecithin, diacetyl tartaric ester of monoglycerides (DATEM), distilled monoglycerides or sodium stearoyl lactylate. The retrogradation properties of the starch at different days, were investigated using a differential scanning calorimeter. Results showed that the addition of emulsifiers did not affect the retrogradation of starch over 5-days.

The effect of dietary fibre on thermal properties of GF bread was investigated by Sabanis and Tzia (2009). Results showed that maize and oat fibre can be added to GF bread with a positive impact on bread's nutritional and sensory properties. Addition of 6 g/100 g maize fibre increased gelatinization temperature compared to control dough. As a general trend it was observed that increasing the fibre concentration in the blend, determined an increase of the on-set temperature. Some authors (Collar, Santos, & Rosell, 2006), have attributed this phenomenon to the fact that dietary fibres, which are a highly water-binding macromolecules, compete with starch for water absorption, limiting starch swelling and gelatinization, thus determining a higher on-set T value. High transition temperatures have also been reported to result from a high degree of starch crystallinity, which provides structural stability and makes the granule more resistant toward gelatinization. Results also showed that an increase in the fibre concentration, has led to a decrease in the gelatinization enthalpy change (ΔHg) values. A decrease in ΔHg, according to the authors, could suggest a synergic interaction between starch and fibre that leads to a more stable structure.

Ronda and Roos (2011) conducted a study on the staling of fresh and frozen GF bread performing a calorimetric analysis on the GF bread crumbs. From this study resulted that at the lowest achievable temperature in common freezers (-28 ℃), the GF bread retained its quality, behaving almost like a fresh bread. At the froze temperature (-14 °C), an acceleration of staling and a considerable loss of bread quality was observed. This was explained observing that less amylopectin recrystallized in bread cooled at -28℃. The study overall confirmed that freezing and frozen storage of GF bread can extent the shelf-life of the product in a satisfactory way, without compromising the bread quality.

The way Xhantan and HPMC, added at different level (2%, 3%, and 5%), affect the physicochemical properties in a model GF dough based on rice cassava, has been studied by Crockett et al. (2011b). The aim of the study was to determine the role of water and flow behaviour upon hydrocolloid addition. Results from Thermogravimetric analysis showed that

hydrocolloid-added dough exhibited a higher water-binding capacity compared to the rice cassava control. As a conclusion, the high methoxy HPMC resulted to be the optimum hydrocolloid in the rice cassava GF dough, but both xanthan and HPMC increased water binding in the dough. Crockett et al. (2011a) also studied the effects of soy protein isolate (added at 1%, 2% and 3%) and egg white solids (added 5%, 10% and 15%) on the physicochemical properties of an HPMC treated gluten-free bread, performing TGA and DSC tests, with the aim to determine the role of water and subsequent flow behaviour upon hydrocolloid addition, as already done in the previous work. The addition of soy protein isolate and egg white solids reduced dough stability by suppressing HPMC functionality, reducing available water, weakening HPMC interactions with the starch matrix and reducing foam stability. The addition of egg white solids, also produced similar antagonistic interaction with HPMC in the HPMC-treated rice cassava dough.

Juszczak et al. (2012) conducted a study to evaluate the effect of inulin with different degrees of polymerization on thermal properties of gluten-free starch-based dough. The addition of inulin did not have an impact on the gelatinization enthalpy (ΔH_g), while it strongly reduced the enthalpies of retrograded amylopectin after storage. Water binding properties of inulin seem to be the key factor, responsible for modification of dough properties, because they influence solvent availability for other constituents of such system.

The influence of modified starches on properties of gluten-free dough and bread was studied by Witczak et al. (2012). Doughs thermograms showed two peaks, associated with the existence of two different starches in the system. No significant effect of modified starch on the onset temperature was observed and only a slight effect of the high amylose corn starch on gelatinization enthalpy was observed. The authors concluded that the optimal composition of the dough formulation requires not only a specific determination of added water, but also an adjustment of hydrocolloids addition, because their effect on dough structure depends on water availability and character of its binding.

Purhagen et al. (2012) studied the anti-staling effect of pre-gelatinized flour (oat and barley) and emulsifier in gluten-free bread. The staling was followed measuring amylopectin retrogradation and the formation of amylose–lipid complex by differential scanning calorimetry. Results showed that adding the emulsifier led to a less retrograded amylopectin as compared to all other recipes. These results were explained considering that one of the mechanisms of the emulsifier is to form complexes with the starch (mainly amylose) and thereby hinder the formation of the amylopectin re-crystallization (retro- gradation) (Richardson et al. 2004).

Another study on the influence of inulin with different degrees of polymerization on physical characteristics and staling rate of GF bread was conducted by Ziobro et al. (2013b) characterising the thermal properties of the GF bread crumb. Results showed a decrease in staling expressed by the amylopectin retrogradation measured by DSC and an accelerated amylopectin recrystallization, induced by the presence of inulin. Inulin preparations with lower degree of polymerization had a slightly stronger effect on all analysed parameters than inulin with higher DP.

The staling characteristics of GF breads based on chestnut and rice flours, has been studied by Demirkesen and Campanella (2014). The effects of chestnut flour and a xanthan–guar gum blend–DATEM has been tested in two different baking conditions: in a conventional and infrared–microwave combination ovens. Results showed that all bread samples exhibited an increase in moisture loss, and retro gradation enthalpy during storage. Baking in different ovens did not affect significantly the retrogradation enthalpies and total crystallinity values of breads. Replacement of rice flour with chestnut flour and addition of xanthan–guar gum blend–DATEM mixture had a positive effect delaying staling of gluten-free breads by decreasing moisture loss, hardness, retrogradation enthalpy, and total mass crystallinity.

Thermal analysis was used by Moreira and Arufe (2015) to investigate the starch transitions of different gluten free flour doughs. GF flour doughs from different maize varieties and from chestnut fruit were analysed. Results showed that the temperatures and enthalpies of the transitions depended on water content, the nature and characteristics (mainly damaged starch) of the starch and the presence of other compounds (mainly lipid and sugars) in the flour doughs.

Authors concluded that starch transitions that take place on flour doughs like gelatinization or amylose melting depend on starch nature. The different nature of starch (average molecular weights of amylose and amylopectin, amylose–amylopectin ratio, etc.) and its interactions with other hydrophilic components (mainly carbohydrates, protein and fibre) modify the available water for starch gelatinization. Consequently, the thermal behaviour of the dough is modified and the temperature ranges for each starch thermal transition must be experimentally determined for each starchy material.

1.8 Infrared analysis applied to the study of GF bread dough

Rheological analysis, TGA and DSC can become even stronger investigation techniques when also used with spectroscopic techniques such as Fourier Transform Infrared Spectroscopy (FTIR). In order to provide chemical and biochemical interpretation for the ingredient interactions during baking and their impact on the final bread quality, an approach based on IR

spectroscopy can be adopted to investigate the complexity of these systems, trying to adapt modern instrumental techniques to the study of bread doughs.

Baking involves heat and mass transfer, inducing physical, chemical and structural changes of bread components (Mondal and Datta, 2008). It involves water evaporation, volume expansion and starch gelatinization. During heating, thermally induced protein denaturation and cross-linking also occur (Arntfield and Murray, 1981). IR spectroscopy is a vibrational spectroscopic technique that allows the study of the chemical composition and molecular structure of heterogeneous foods and biological materials and can be used to analyse the secondary structures and conformations of protein/polysaccharides based on the characteristic absorption bands of specific functional groups contained within these biopolymers. Because of the complexity of food systems, their spectra are composed of broad overlapped bands. For this reason, mathematical procedures are used to resolve the overlapped and hidden peaks.

FTIR is one of the earliest experimental methods applied to the evaluation of the secondary structure of polypeptides and proteins (Byler et al., 1986; Krimm et al., 1986) and the use of spectroscopy in the study of food systems is becoming more common. Even if there is not yet a wide literature on it, especially regarding the study of baked goods, there are some studies on gluten-containing bread. Gluten and its interactions with other ingredients in a formulation have been studied by Sivam et al., (2012) who explored the interactions between blackcurrant polyphenols, pectin and wheat biopolymers in model breads. Results from FT-IR analysis revealed that replacing flour with starch and gluten, adding blackcurrant polyphenols extract and pectin, or removing bread components (oil, salt or the mixture of yeast and sugar), caused changes in the bread gluten conformations related to Amide I and II bands.

Sivam et al. (2013) also studied wheat breads formulations, fortified with fibre and polyphenols, investigating the interactions among added pectin polysaccharides, polyphenols with wheat proteins in different bread formulations prepared varying the amount of water. Results showed that adding pectin and polyphenols caused changes in the molecular conformations and polymer structure of wheat gluten and starch in the finished breads, especially in the secondary protein conformation (Amides I and II) as revealed by FTIR Spectroscopy. An increase of water content during dough formulation also affected secondary protein conformation. The Treated breads gained β-sheets in the protein's secondary conformation at the expense of β-turns, and contained more unordered conformations especially in the presence of berry polyphenols.

As far as GF bread is concerned, very little literature is available in this field. A study on acidic extruded rice flour and its influence on the qualities of GF bread has been carried out by Clerici et al. (2009). The acidic extruded rice flour was produced in a single- screw extruder,

varying the extrusion temperature and lactic acid concentration. IR spectroscopy showed that no bands were found for esters (1740 cm 1) in the acidic extruded rice flour, which could indicate that no new bonds were formed between the rice flour and the lactic acid.

Demirkesen and Campanella (2013) evaluated the effects of chestnut flour and a xanthan–guar gum blend–DATEM mixture on staling of GF rice breads baked in conventional and infrared–microwave combination ovens. FT-IR spectra showed that the integrated area of peaks around 1041 cm^{-1} and 1150 cm^{-1} wave lengths, which are related to the structure of starch retrogradation, increased with storage time. FT-IR measurements showed that both starch and water played important roles in the staling mechanism.

1.9 Starting points for this research

The topic of my research will be the study of GF bread model systems, being GF bread a product belonging to the category of medical foods, which can be considered a particular subclass of LP/PF foods, because of the protein nature of the gluten constituent polymers. Despite major improvements and achievements in the field of the production of these baked goods, much remains to be discovered in relation to the role and the structuring mechanism of the constituents of GF bread, and in particular those substances, such as non-starch hydrocolloids and non-glutinic proteins, which are used to replace gluten in order to achieve a soft honeycombed structure, comparable to that of wheat products. However, the structural problem is not the only one. From the vast literature analysis, it emerges that another aspect of importance equal to the structural one, if not greater, remains the nutritional one. The use of high-fibre products is becoming popular to try to limit excessive carbohydrate intake that can easily be assimilated, leading to other pathologies such as diabetes in celiac disease and to increase the fibre intake in the diet of celiac patients, The fibres, even if it is not yet clear, also seem to have a positive effect on the structuring of GF bakery products. In the light of what emerged from literature analysis, my work will focus on the optimization of GF bread formulations obtained from materials that, besides having a great potential in structuring terms, can bring health benefits to the consumers.

A good starting point for this research will be to compare the behaviour of two different types of bread formulations, to test the ability of innovative GF flours, based on pseudo cereals, resistant starch and cellulose hydrocolloids, in GF bread making. The effects of vegetable proteins, as a substitute for animal proteins, will also be studied, with the aim of maximizing their structuring potential ability by reducing their content through the help of enzymes that have the ability to promote the protein crosslink. The optimization is mainly based on a

rheological study combined with other investigation techniques such as thermal analysis and infrared spectroscopy.

References

- Acs, E., Kovacs, Z., & Matuz, J. (1997). Possibilities of producing low-protein, gluten-free bread. 1. Structure formation. *Novenytermeles*, *46*(3), 227-234.

- Afolabi, P. R., F. Jahoor, N. R. Gibson & A. A. Jackson (2004) Response of hepatic proteins to the lowering of habitual dietary protein to the recommended safe level of intake. *American Journal of Physiology-Endocrinology and Metabolism*, 287, E327-E330.

- Aguilar, N., Albanell, E., Miñarro, B., & Capellas, M. (2015). Chickpea and tiger nut flours as alternatives to emulsifier and shortening in gluten-free bread. *LWT-Food Science and Technology*, *62*(1), 225-232.

- Ahlborn, G. J., Pike, O. A., Hendrix, S. B., Hess, W. M., & Huber, C. S. (2005). Sensory, mechanical, and microscopic evaluation of staling in low-protein and gluten-free breads. *Cereal chemistry*, *82*(3), 328-335.

- Ahring, K., Bélanger-Quintana, A., Dokoupil, K., Ozel, H. G., Lammardo, A. M., MacDonald, A., Motzfeldt K, Nowacka M, Robert M, van Rijn M. (2009). Dietary management practices in phenylketonuria across European centers. *Clinical nutrition*, *28*(3), 231-236.

- Allred, C. R. G., Voss, A. C., Finn, S. C., & McCamish, M. A. (1996). Malnutrition and clinical outcomes: the case for medical nutrition therapy. *Journal of the American Dietetic Association*, *96*(4), 361-369.

- Alvarez-Jubete, L., Arendt, E. K., & Gallagher, E. (2009). Nutritive value and chemical composition of pseudocereals as gluten-free ingredients. *International Journal of Food Sciences and Nutrition*, *60*(sup4), 240-257.

- Anderson, L. A., McMillan, S. A., Watson, R. G. P., Monaghan, P., Gavin, A. T., Fox, C., & Murray, L. J. (2007). Malignancy and mortality in a population-based cohort of patients with coeliac disease or 'gluten sensitivity'. *World journal of gastroenterology*, *13*(1), 146.

- Andersson, H., Öhgren, C., Johansson, D., Kniola, M., & Stading, M. (2011). Extensional flow, viscoelasticity and baking performance of gluten-free zein-starch doughs supplemented with hydrocolloids. *Food hydrocolloids*, *25*(6), 1587-1595.

▪ Anton, A. A., & Artfield, S. D. (2008). Hydrocolloids in gluten-free breads: a review. *International journal of food sciences and nutrition*, *59*(1), 11-23.

▪ Aparicio, M., Bellizzi, V., Chauveau, P., Cupisti, A., Ecder, T., Fouque, Garneata, L., Lin, S., Mitch, W.E., Teplan, V. and Zakar, G. (2012). Keto acid therapy in pre-dialysis chronic kidney disease patients: final consensus. *Journal of renal nutrition*, 22(2), S22-S24.

▪ (2012). Keto acid therapy in pre-dialysis chronic kidney disease patients: final consensus. *Journal of renal nutrition*, *22*(2), S22-S24.

▪ Aparicio, M., Chauveau, P., De Précigout, V., Bouchet, J. L., Lasseur, C., & Combe, C. (2000). Nutrition and outcome on renal replacement therapy of patients with chronic renal failure treated by a supplemented very low protein diet. *Journal of the American Society of Nephrology*, *11*(4), 708-716.

▪ Apovian, C. M. (2004). Sugar-sweetened soft drinks, obesity, and type 2 diabetes. *Jama*, *292*(8), 978-979.

▪ Arntfield, S. D., & Murray, E. D. (1981). The influence of processing parameters on food protein functionality I. Differential scanning calorimetry as an indicator of protein denaturation. *Canadian Institute of Food Science and Technology Journal*, 14(4), 289-294.

▪ Bakke, A. & Z. Vickers (2007) Consumer liking of refined and whole wheat breads. *Journal of Food Science*, 72, S473-S480.

▪ Bellizzi, V., A. Cupisti, F. Locatelli, P. Bolasco, G. Brunori, G. Cancarini, S. Caria, L. De Nicola, B. R. Di Iorio & L. Di Micco (2016) Low-protein diets for chronic kidney disease patients: the Italian experience. *BMC nephrology*, 17, 77.

▪ Birt, D.F., Boylston, T., Hendrich, S., Jane, J.L., Hollis, J., Li, L., McClelland, J., Moore, S., Phillips, G.J., Rowling, M. and Schalinske, K., (2013). Resistant starch: promise for improving human health. *Advances in Nutrition: An International Review Journal*, *4*(6), 587-601.

▪ Blau, N., Belanger-Quintana, A., Demirkol, M., Feillet, F., Giovannini, M., Macdonald, A.,Trefz FK, van Spronsen F, (2009). Management Of Phenylketonuria (pku) In Europe: Survey Results From 19 Countries. *Acta Paediatrica*, *98*, 144-145.

▪ Bowersox, J., N. C. D. Panel & N. C. D. Panel (2001) National Institutes of Health Consensus Development Conference Statement: Phenylketonuria: Screening and Management, October 16-18, 2000. *Pediatrics*, 108, 972-982.

▪ Briani, C., Samaroo, D., & Alaedini, A. (2008). Celiac disease: from gluten to autoimmunity. *Autoimmunity reviews*, *7*(8), 644-650.

- Burešová, I., Kráčmar, S., Dvořáková, P., & Středa, T. (2014). The relationship between rheological characteristics of gluten-free dough and the quality of biologically leavened bread. *Journal of Cereal Science*, *60*(2), 271-275.

- Burešová, I., Masaříková, L., Hřivna, L., Kulhanová, S., & Bureš, D. (2016). The comparison of the effect of sodium caseinate, calcium caseinate, carboxymethyl cellulose and xanthan gum on rice-buckwheat dough rheological characteristics and textural and sensory quality of bread. *LWT-Food Science and Technology*, *68*, 659-666.

- Byler, D. M., & Susi, H. (1986). Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers*, 25(3), 469-487.

- Carey, M., & Gillespie, S. (1995). Position of The American Dietic Association: Cost-effectiveness of medical nutrition therapy. *Journal of the Academy of Nutrition and Dietetics*, *95*(1), 88.

- Catassi, C., & Fasano, A. (2008). Celiac disease. *Current opinion in gastroenterology*, *24*(6), 687-691.

- Catassi, C., Fabiani, E., Iacono, G., D'agate, C., Francavilla, R., Biagi, F., Volta U, Accomando S, Picarelli A, De Vitis I, Pianelli G. (2007). A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease. *The American journal of clinical nutrition*, 85(1), 160-166.

- Cato, L., Rafael, L. G. B., Gan, J., & Small, D. M. (2002). The use of rice flour and hydrocolloid gums for gluten free breads. In *Proceedings of the 51st Australian cereal chemistry conference* (pp. 304-308).

- Clerici, M. T. P. S., Airoldi, C., & El-Dash, A. A. (2009). Production of acidic extruded rice flour and its influence on the qualities of gluten-free bread. LWT-*Food Science and Technology*, 42(2), 618-623.

- Collar, C., Santos, E., & Rosell, C. M. (2006). Significance of dietary fiber on the viscometric pattern of pasted and gelled flour-fiber blends. *Cereal Chemistry*, 83(4), 370-376.

- Coresh, J., Astor, B. C., Greene, T., Eknoyan, G., & Levey, A. S. (2003). Prevalence of chronic kidney disease and decreased kidney function in the adult US population: Third National Health and Nutrition Examination Survey. *American journal of kidney diseases*, *41*(1), 1-12.

- Crockett, R., Ie, P., & Vodovotz, Y. (2011a). Effects of soy protein isolate and egg white solids on the physicochemical properties of gluten-free bread. *Food Chemistry*, 129(1), 84-91.

- Crockett, R., Ie, P., & Vodovotz, Y. (2011b). How Do Xanthan and Hydroxypropyl Methylcellulose Individually Affect the Physicochemical Properties in a Model Gluten-Free Dough?. *Journal of food science*, 76(3).

- D'Alessandro, C., Rossi, A., Innocenti, M., Ricchiuti, G., Bozzoli, L., Sbragia, G., Meola, M. and Cupisti, A. (2013). Dietary protein restriction for renal patients: don't forget protein-free foods. *Journal of Renal Nutrition*, *23*(5), 367-371.

- Demirkesen, I., Campanella, O. H., Sumnu, G., Sahin, S., & Hamaker, B. R. (2014). A study on staling characteristics of gluten-free breads prepared with chestnut and rice flours. *Food and bioprocess technology*, 7(3), 806-820.

- Demirkesen, I., Mert, B., Sumnu, G., & Sahin, S. (2010). Rheological properties of gluten-free bread formulations. *Journal of food Engineering*, *96*(2), 295-303.

- Deora, N. S., Deswal, A., & Mishra, H. N. (2015). Functionality of alternative protein in gluten-free product development. *Revista de Agaroquimica y Tecnologia de Alimentos*, *21*(5), 364-379.

- Diez Poza, O. (2002). Transglutaminase in baking applications. *Cereal Foods World*, *47*(3), 93-95.

- Dobraszczyk, B. J., Campbell, G. M., & Gan, Z. (2001). Bread: a unique food. Aspen Publishers.

- Fessas, D. & A. Schiraldi (2001) Water properties in wheat flour dough - I: Classical thermogravimetry approach. *Food Chemistry*, 72, 237-244.

- Fessas, D., & Schiraldi, A. (2000). Starch Gelatinization Kinetics in Bread Dough. DSC investigations on simulated baking processes. *Journal of Thermal Analysis and Calorimetry*, 61(2), 411-423.

- Fessas, D., Signorelli, M., Pagani, A., Mariotti, M., Iametti, S., & Schiraldi, A. (2008). Guidelines for buckwheat enriched bread: thermal analysis approach. *Journal of Thermal Analysis and Calorimetry*, 91(1), 9-16.

- Fouque, D., & Laville, M. (2009). Low protein diets for chronic kidney disease in non-diabetic adults. *The Cochrane Library*.

- Fouque, D., Wang, P., Laville, M., & Boissel, J. P. (2000). Low protein diets delay end-stage renal disease in non-diabetic adults with chronic renal failure. *Nephrology dialysis transplantation*, *15*(12), 1986-1992.

- Fujioka, K. (2002). Management of obesity as a chronic disease: nonpharmacologic, pharmacologic, and surgical options. *Obesity*, *10*(S12).

- Gallagher, E., Gormley, T. R., & Arendt, E. K. (2003). Crust and crumb characteristics of gluten free breads. *Journal of Food Engineering*, 56(2), 153-161.

▪ Gallagher, E., Gormley, T. R., & Arendt, E. K. (2004a). Recent advances in the formulation of gluten-free cereal-based products. *Trends in Food Science & Technology*, *15*(3), 143-152.

▪ Gallagher, E., McCarthy, D., Gormley, R. T., & Arendt, E. (2004b). Improving the quality of gluten-free products. Teagasc.

▪ Giovannetti, S. (1986). Low protein diet in chronic uremia: a historical survey. In *Low Protein Diet and Progression of Chronic Renal Failure* (Vol. 53, pp. 1-6). Karger Publishers.

▪ Giovannetti, S., & Maggiore, Q. (1964). A low-nitrogen diet with proteins of high biological value for severe chronic uraemia. *The Lancet*, *283*(7341), 1000-1003.

▪ Giovannini, M.., Verduci, E., Salvatici, E., Fiori, L., & Riva, E. (2007). Phenylketonuria: dietary and therapeutic challenges. *Journal of inherited metabolic disease*, *30*(2), 145-152.

▪ Goesaert, H., Brijs, K., Veraverbeke, W. S., Courtin, C. M., Gebruers, K., & Delcour, J. A. (2005). Wheat flour constituents: how they impact bread quality, and how to impact their functionality. *Trends in food science & technology*, 16(1), 12-30.

▪ Goesaert, H., Courtin, C. M., & Delcour, J. A. (2008). Use of enzymes in the production of cereal-based functional foods and food ingredients. *Gluten-free cereal products and beverages. Elsevier, Burlington*, 237-265.

▪ Greve, L. C., Wheeler, M. D., Green-Burgeson, D. K., & Zorn, E. M. (1994). Breast-feeding in the management of the newborn with phenylketonuria: a practical approach to dietary therapy. *Journal of the American Dietetic Association*, *94*(3), 305-309.

▪ Gujral, H. S., & Rosell, C. M. (2004). Functionality of rice flour modified with a microbial transglutaminase. *Journal of Cereal Science*, *39*(2), 225-230.

▪ Gujral, H. S., Guardiola, I., Carbonell, J. V., & Rosell, C. M. (2003). Effect of cyclodextrinase on dough rheology and bread quality from rice flour. *Journal of Agricultural and Food Chemistry*, *51*(13), 3814-3818.

▪ Hager, A. S., & Arendt, E. K. (2013). Influence of hydroxypropylmethylcellulose (HPMC), xanthan gum and their combination on loaf specific volume, crumb hardness and crumb grain characteristics of gluten-free breads based on rice, maize, teff and buckwheat. *Food Hydrocolloids*, *32*(1), 195-203.

▪ Hager, A. S., Ryan, L. A., Schwab, C., Gänzle, M. G., O'Doherty, J. V., & Arendt, E. K. (2011). Influence of the soluble fibres inulin and oat β-glucan on quality of dough and bread. *European Food Research and Technology*, *232*(3), 405-413.

- Hager, M., A. Hutchins, ADA & ADA (2003) Position of the American Dietetic Association: Integration of medical nutrition therapy and pharmacotherapy. *Journal of the American Dietetic Association*, 103, 1363-1370.

- Haque, A., & Morris, E. R. (1994). Combined use of ispaghula and HPMC to replace or augment gluten in breadmaking. *Food Research International*, *27*(4), 379-393.

- Harsha, D. W., Lin, P. H., Obarzanek, E. V. A., Karanja, N. M., Moore, T. J., Caballero, B., & DASH Collaborative Research Group. (1999). Dietary approaches to stop hypertension: a summary of study results. *Journal of the American Dietetic Association*, *99*(8), S35-S39.

- He, H., & Hoseney, R. C. (1991). Gas retention of different cereal flours. *Cereal Chemistry*, 68(4), 334-336.

- Horstmann, S. W., Foschia, M., & Arendt, E. K. (2017). Correlation analysis of protein quality characteristics with gluten-free bread properties. *Food & Function*.

- Houben, A., Höchstötter, A., & Becker, T. (2012). Possibilities to increase the quality in gluten-free bread production: an overview. *European Food Research and Technology*, *235*(2), 195-208.

- Juszczak, L., Witczak, T., Ziobro, R., Korus, J., Cieślik, E., & Witczak, M. (2012). Effect of inulin on rheological and thermal properties of gluten-free dough. *Carbohydrate polymers*, 90(1), 353-360.

- Kang, M. Y., Choi, Y. H., & Choi, H. C. (1997). Effects of gums, fats and glutens adding on processing and quality of milled rice bread. *Korean Journal of Food Science and Technology*, *29*(4), 700-704.

- Kasiske, B. L., Lakatua, J. D., Ma, J. Z., & Louis, T. A. (1998). A meta-analysis of the effects of dietary protein restriction on the rate of decline in renal function. *American Journal of Kidney Diseases*, *31*(6), 954-961.

- Keetels, C. J. A. M., Visser, K. A., Van Vliet, T., Jurgens, A., & Walstra, P. (1996). Structure and mechanics of starch bread. *Journal of Cereal Science*, *24*(1), 15-26.

- Kemppainen, T., Kröger, H., Janatuinen, E., Arnala, I., Kosma, V. M., Pikkarainen, P., Julkunen, R., Jurvelin, J., Alhava, E. and Uusitupa, M., (1999). Osteoporosis in adult patients with celiac disease. *Bone*, *24*(3), 249-255.

- Khatkar, B. S., Bell, A. E., & Schofield, J. D. (1995). The dynamic rheological properties of glutens and gluten sub-fractions from wheats of good and poor bread making quality. *Journal of Cereal Science*, *22*(1), 29-44.

- Khatkar, B. S., Fido, R. J., Tatham, A. S., & Schofield, J. D. (2002). Functional properties of wheat gliadins. I. Effects on mixing characteristics and bread making quality. *Journal of Cereal Science*, 35(3), 299-306.

- Korus, J., M., Ziobro, R., & Juszczak, L. (2009). The impact of resistant starch on characteristics of gluten-free dough and bread. *Food Hydrocolloids*,*23*(3), 988-995.

- Korus, J., Witczak, M., Ziobro, R., & Juszczak, L. (2017). Hemp (Cannabis sativa subsp. sativa) flour and protein preparation as natural nutrients and structure forming agents in starch based gluten-free bread. *LWT-Food Science and Technology*.

- Krimm, S., & Bandekar, J. (1986). Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins. *Advances in protein chemistry*, 38, 181-364.

- Kulp, K., Hepburn, F. N., & Lehmann, T. (1974). Preparation of bread without gluten. *Bakers digest*.

- Lazaridou, A., & Biliaderis, C. G. (2009). Gluten-free doughs: rheological properties, testing procedures–methods and potential problems. *Gluten-free food science and technology*, 52-82.

- Lazaridou, A., Duta, D., Papageorgiou, M., Belc, N., & Biliaderis, C. G. (2007). Effects of hydrocolloids on dough rheology and bread quality parameters in gluten-free formulations. *Journal of food engineering*, *79*(3), 1033-1047.

- Lee, A., & Newman, J. M. (2003). Celiac diet: its impact on quality of life. *Journal of the American Dietetic Association*, *103*(11), 1533-1535.

- Lee, M. H., & Lee, Y. T. (2006). Properties of gluten-free rice breads using different rice flours prepared by dry, wet and semi-wet milling. *Food Engineering Progress*.

- Levey, A. S., Coresh, J., Balk, E., Kausz, A. T., Levin, A., Steffes, M. W., Hogg, R.J., Perrone, R.D., Lau, J. and Eknoyan, G (2003). National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Annals of internal medicine*, *139*(2), 137-147.

- Levey, A. S., De Jong, P. E., Coresh, J., Nahas, M. E., Astor, B. C., Matsushita, K., Gansevoort, R.T., Kasiske, B.L. and Eckardt, K.U. (2011). The definition, classification, and prognosis of chronic kidney disease: a KDIGO Controversies Conference report. *Kidney international*, *80*(1), 17-28.

- Lorig, K. R., Sobel, D. S., Stewart, A. L., Brown Jr, B. W., Bandura, A., Ritter, P., Gonzalez, V.M., Laurent, D.D. and Holman, H.R. (1999). Evidence suggesting that a chronic disease self-management program can improve health status while reducing hospitalization: a randomized trial. *Medical care*, *37*(1), 5-14.

- MacKay, L. L., Addis, T., & MacKay, E. M. (1938). The degree of compensatory renal hypertrophy following unilateral nephrectomy. *Journal of Experimental Medicine*, *67*(4), 515-519.

- Mahmoud, R. M., Yousif, E. I., Gadallah, M. G. E., & Alawneh, A. R. (2013). Formulations and quality characterization of gluten-free Egyptian balady flat bread. *Annals of Agricultural Sciences*, *58*(1), 19-25.

- Mancebo, C. M., San Miguel, M. Á., Martínez, M. M., & Gómez, M. (2015). Optimisation of rheological properties of gluten-free doughs with HPMC, psyllium and different levels of water. *Journal of Cereal Science*, *61*, 8-15.

- Marco, C., & Rosell, C. M. (2008). Breadmaking performance of protein enriched, gluten-free breads. *European Food Research and Technology*, *227*(4), 1205-1213.

- Mariotti, M., Lucisano, M., Pagani, M. A., & Ng, P. K. (2009). The role of corn starch, amaranth flour, pea isolate, and Psyllium flour on the rheological properties and the ultrastructure of gluten-free doughs. *Food Research International*, *42*(8), 963-975.

- Marston, K., Khouryieh, H., & Aramouni, F. (2016). Effect of heat treatment of sorghum flour on the functional properties of gluten-free bread and cake. *LWT-Food Science and Technology*, *65*, 637-644.

- Martínez, M. M., & Gómez, M. (2017). Rheological and microstructural evolution of the most common gluten-free flours and starches during bread fermentation and baking. *Journal of Food Engineering*, 197, 78-86.

- Marzocco, S., Dal Piaz, F., Di Micco, L., Torraca, S., Sirico, M. L., Tartaglia, D., Autore, G. and Di Iorio, B. (2013). Very low protein diet reduces indoxyl sulfate levels in chronic kidney disease. *Blood purification*, *35*(1-3), 196-201.

- Masure, H. G., Fierens, E., & Delcour, J. A. (2016). Current and forward looking experimental approaches in gluten-free bread making research. *Journal of Cereal Science*, *67*, 92-111.

- Matos Segura ME, Rosell CM (2011) Chemical composition and starch digestibility of different gluten-free breads. Plant Foods Hum Nutr 66(3):224–230

- Matos, M. E., & Rosell, C. M. (2013). Quality indicators of rice-based gluten-free bread-like products: relationships between dough rheology and quality characteristics. *Food and Bioprocess Technology*, *6*(9), 2331-2341.

- Matveev, Y., V. Grinberg & V. Tolstoguzov (2000) The plasticizing effect of water on proteins, polysaccharides and their mixtures. Glassy state of biopolymers, food and seeds. *Food Hydrocolloids*, 14, 425-437.

- McAdam, S. N., & Sollid, L. M. (2000). Getting to grips with gluten. *Gut*, *47*(6), 743-745.

- McCarthy, D. F., Gallagher, E., Gormley, T. R., Schober, T. J., & Arendt, E. K. (2005). Application of response surface methodology in the development of gluten-free bread. *Cereal chemistry*, *82*(5), 609-615.

- Millward, D. J., & Jackson, A. A. (2004). Protein/energy ratios of current diets in developed and developing countries compared with a safe protein/energy ratio: implications for recommended protein and amino acid intakes. *Public health nutrition*, *7*(3), 387-405.

- Mircescu, G., Gârneaţă, L., Stancu, S. H., & Căpuşă, C. (2007). Effects of a supplemented hypoproteic diet in chronic kidney disease. *Journal of Renal Nutrition*, *17*(3), 179-188.

- Mitch, W. E. (1991). Dietary protein restriction in chronic renal failure: nutritional efficacy, compliance, and progression of renal insufficiency. *Journal of the American Society of Nephrology*, *2*(4), 823-831.

- Mondal, A., & Datta, A. K. (2008). Bread baking–a review. Journal of Food Engineering, 86(4), 465-474.

- Moore, M. M., Heinbockel, M., Dockery, P., Ulmer, H. M., & Arendt, E. K. (2006). Network formation in gluten-free bread with application of transglutaminase. *Cereal chemistry*, *83*(1), 28-36.

- Moore, M. M., Schober, T. J., Dockery, P., & Arendt, E. K. (2004). Textural comparisons of gluten-free and wheat-based doughs, batters, and breads. *Cereal Chemistry*, *81*(5), 567-575.

- Morais, E. C., Cruz, A. G., Faria, J. A. F., & Bolini, H. M. A. (2014). Prebiotic gluten-free bread: Sensory profiling and drivers of liking. *LWT-Food Science and Technology*, *55*(1), 248-254.

- Moreira, R., Chenlo, F., & Arufe, S. (2015). Starch transitions of different gluten free flour doughs determined by dynamic thermal mechanical analysis and differential scanning calorimetry. *Carbohydrate polymers*, 127, 160-167.

- Moreira, R., Chenlo, F., & Torres, M. D. (2011). Rheological properties of commercial chestnut flour doughs with different gums. *International journal of food science & technology*, *46*(10), 2085-2095.

- Moreira, R., Chenlo, F., & Torres, M. D. (2013). Effect of chia (Sativa hispanica L.) and hydrocolloids on the rheology of gluten-free doughs based on chestnut flour. *LWT-Food Science and Technology*, *50*(1), 160-166.

- Motarjemi, Y. (1991). Codex Committee on nutrition and foods for special dietary uses. *Food Control*, *2*(3), 185.

- Mudgil, D., & Barak, S. (2013). Composition, properties and health benefits of indigestible carbohydrate polymers as dietary fiber: a review. *International journal of biological macromolecules*, *61*, 1-6.

- Nestle, M. (2003). Increasing portion sizes in American diets: more calories, more obesity. *Journal of the Academy of Nutrition and Dietetics*, *103*(1), 39.

- Niewinski, M. M. (2008). Advances in celiac disease and gluten-free diet. *Journal of the American Dietetic Association*, *108*(4), 661-672.

- Nishita, K.D., Roberts, R.L., Bean, M.M. (1976). Development of a yeast leavened rice bread formula. Cereal Chemistry, 53, 626-635.

- Nunes, M. H. B., Moore, M. M., Ryan, L. A., & Arendt, E. K. (2009). Impact of emulsifiers on the quality and rheological properties of gluten-free breads and batters. *European Food Research and Technology*, 228(4), 633-642.

- Onyango, C., Unbehend, G., & Lindhauer, M. G. (2009). Effect of cellulose-derivatives and emulsifiers on creep-recovery and crumb properties of gluten-free bread prepared from sorghum and gelatinised cassava starch. *Food Research International*, *42*(8), 949-955.

- Özboy, Ö. (2002). Development of corn starch-gum bread for phenylketonuria patients. *Molecular Nutrition & Food Research*, *46*(2), 87-91.

- Pastors, J. G., Warshaw, H., Daly, A., Franz, M., & Kulkarni, K. (2002). The evidence for the effectiveness of medical nutrition therapy in diabetes management. *Diabetes care*, *25*(3), 608-613.

- Petrofsky, K. E., & Hoseney, R. C. (1995). Rheological properties of dough made with starch and gluten from several cereal sources. *Cereal Chemistry*, *72*(1), 53-57.

- Piccoli, G. B., Capizzi, I., Vigotti, F. N., Leone, F., D'Alessandro, C., Giuffrida, D., Nazha, M., Roggero, S., Colombi, N., Mauro, G. and Castelluccia, N. (2016). Low protein diets in patients with chronic kidney disease: a bridge between mainstream and complementary-alternative medicines. *BMC nephrology*, *17*(1), 76.

- Purhagen, J. K., Sjöö, M. E., & Eliasson, A. C. (2012). The anti-staling effect of pre-gelatinized flour and emulsifier in gluten-free bread. European *Food Research and Technology*, 235(2), 265-276.

- Renzetti, S., Dal Bello, F., & Arendt, E. K. (2008). Microstructure, fundamental rheology and baking characteristics of batters and breads from different gluten-free

flours treated with a microbial transglutaminase. *Journal of Cereal Science*, *48*(1), 33-45.

- Ribotta, P. D., Ausar, S. F., Morcillo, M. H., Pérez, G. T., Beltramo, D. M., & León, A. E. (2004). Production of gluten-free bread using soybean flour. *Journal of the Science of Food and Agriculture*, *84*(14), 1969-1974.

- Richardson, G., Kidman, S., Langton, M., & Hermansson, A. M. (2004). Differences in amylose aggregation and starch gel formation with emulsifiers. *Carbohydrate Polymers*, 58(1), 7-13.

- Rissanen, T. H., Voutilainen, S., Virtanen, J. K., Venho, B., Vanharanta, M., Mursu, J., & Salonen, J. T. (2003). Low intake of fruits, berries and vegetables is associated with excess mortality in men: the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study. *The Journal of nutrition*, *133*(1), 199-204.

- Rojas, J. A., Rosell, C. M., & De Barber, C. B. (1999). Pasting properties of different wheat flour-hydrocolloid systems. *Food hydrocolloids*, *13*(1), 27-33.

- Ronda, F., & Roos, Y. H. (2011). Staling of fresh and frozen gluten-free bread. *Journal of cereal science*, 53(3), 340-346.

- Ronda, F., Villanueva, M., & Collar, C. (2014). Influence of acidification on dough viscoelasticity of gluten-free rice starch-based dough matrices enriched with exogenous protein. *LWT- Food Science and Technology*, 59(1), 12-20.

- Rosell, C. M. (2009). Enzymatic manipulation of gluten-free breads. *Gluten-free food science and technology*, 83-98.

- Rosell, C. M., Collar, C., & Haros, M. (2007). Assessment of hydrocolloid effects on the thermo-mechanical properties of wheat using the Mixolab. *Food Hydrocolloids*, *21*(3), 452-462.

- Rosell, C. M., Rojas, J. A., & De Barber, C. B. (2001). Influence of hydrocolloids on dough rheology and bread quality. Food hydrocolloids, 15(1), 75-81.

- Sabanis, D., Lebesi, D., & Tzia, C. (2009). Effect of dietary fibre enrichment on selected properties of gluten-free bread. *LWT-Food Science and Technology*, *42*(8), 1380-1389.

- Sapone, A., Lammers, K. M., Casolaro, V., Cammarota, M., Giuliano, M. T., De Rosa, M., Stefanile R, Mazzarella G, Tolone C, Russo M.I, Esposito P. (2011). Divergence of gut permeability and mucosal immune gene expression in two gluten-associated conditions: celiac disease and gluten sensitivity. *BMC medicine*, *9*(1), 23.

- Schiraldi, A., & Fessas, D. (2003). Classical and Knudsen thermogravimetry to check states and displacements of water in food systems. *Journal of thermal analysis and calorimetry*, 71(1), 225-235.

- Schiraldi, A., Piazza, L., Brenna, O., & Vittadini, E. (1996). Structure and properties of bread dough and crumb: calorimetric, rheological and mechanical investigations on the effects produced by hydrocolloids, pentosans and soluble proteins. *Journal of Thermal Analysis and Calorimetry*, 47(5), 1339-1360.

- Schober, T. J., Bean, S. R., & Boyle, D. L. (2007). Gluten-free sorghum bread improved by sourdough fermentation: biochemical, rheological, and microstructural background. *Journal of Agricultural and Food Chemistry*, *55*(13), 5137-5146.

- Schober, T. J., Bean, S. R., Tilley, M., Smith, B. M., & Ioerger, B. P. (2011). Impact of different isolation procedures on the functionality of zein and kafirin. *Journal of cereal science*, *54*(2), 241-249.

- Schober, T. J., Messerschmidt, M., Bean, S. R., Park, S. H., & Arendt, E. K. (2005). Gluten-free bread from sorghum: quality differences among hybrids. *Cereal chemistry*, *82*(4), 394-404.

- Schoenlechner, R., Mandala, I., Kiskini, A., Kostaropoulos, A., & Berghofer, E. (2010). Effect of water, albumen and fat on the quality of gluten-free bread containing amaranth. *International journal of food science & technology*, *45*(4), 661-669.

- Schofield, J. D. (1994). Wheat proteins: structure and functionality in milling and breadmaking. In *Wheat* (pp. 73-106). Springer US.

- Schweizer, J. J., Oren, A., & Mearin, M. L. (2001). Cancer in children with celiac disease: a survey of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition. *Journal of pediatric gastroenterology and nutrition*, *33*(1), 97-100.

- Sethi, G. R., Singhal, K. K., Puri, A. S., & Mantan, M. (2011). Benefit of gluten-free diet in idiopathic pulmonary hemosiderosis in association with celiac disease. *Pediatric pulmonology*, *46*(3), 302-305.

- Shan, L., Molberg, Ø., Parrot, I., Hausch, F., Filiz, F., Gray, G. M., ... & Khosla, C. (2002). Structural basis for gluten intolerance in celiac sprue. *Science*, *297*(5590), 2275-2279.

- Singh, H., & MacRitchie, F. (2004). Changes in proteins induced by heating gluten dispersions at high temperature. *Journal of Cereal Science*, *39*(2), 297-301.

- Singh, R. H., Cunningham, A. C., Mofidi, S., Douglas, T. D., Frazier, D. M., Hook, D.G., Jeffers, L., McCune, H., Moseley, K.D., Ogata, B. and Pendyal, S. (2016). Updated, web-based nutrition management guideline for PKU: An evidence and consensus based approach. *Molecular genetics and metabolism*, *118*(2), 72-83.

- Singh, R. H., Rohr, F., Frazier, D., Cunningham, A., Mofidi, S., Ogata, B., Splett, P.L., Moseley, K., Huntington, K., Acosta, P.B. and Vockley, J (2014). Recommendations for

the nutrition management of phenylalanine hydroxylase deficiency. *Genetics in Medicine*, *16*(2), 121.

- Sivam, A. S., Sun-Waterhouse, D., Perera, C. O., & Waterhouse, G. I. N. (2012). Exploring the interactions between blackcurrant polyphenols, pectin and wheat biopolymers in model breads; a FTIR and HPLC investigation. *Food Chemistry*, 131(3), 802-810.

- Sivam, A. S., Sun-Waterhouse, D., Perera, C. O., & Waterhouse, G. I. N. (2013). Application of FT-IR and Raman spectroscopy for the study of biopolymers in breads fortified with fibre and polyphenols. *Food Research International*, 50(2), 574-585.

- Sivaramakrishnan, H. P., Senge, B., & Chattopadhyay, P. K. (2004). Rheological properties of rice dough for making rice bread. *Journal of Food Engineering*, *62*(1), 37-45.

- Smith, I., Cockburn, F., Barwell, B., Brenton, D., Chapple, J., Clark, B., Curzon, G., Davidson, D., Heeley, A., Laing, S. and Listercheese, I. (1993). Recommendations on the dietary management of phenylketonuria. *Arch Dis Child*, *68*(3), 426-427.

- Tajik, P. (2014). Celiac disease overview in children by focused on Iranian studies. *International Journal of Celiac Disease*, *2*(4), 121-125.

- Thompson, T. (2000). Folate, iron, and dietary fiber contents of the gluten-free diet. *Journal of the American Dietetic Association*, *100*(11), 1389-1396.

- Tsatsaragkou, K., Gounaropoulos, G., & Mandala, I. (2014). Development of gluten free bread containing carob flour and resistant starch. *LWT-Food Science and Technology*, *58*(1), 124-129.

- van Berge-Henegouwen, G. P., & Mulder, C. J. (1993). Pioneer in the gluten free diet: Willem-Karel Dicke 1905-1962, over 50 years of gluten free diet. *Gut*, *34*(11), 1473.

- Van Bockstaele, F., Batharrai, M., Vervoort, S., & Dewettinck, K. (2016). Interactions between wheat starch, hydroxypropyl methyl-cellulose (HPMC) and psyllium seed husk in gluten-free breadmaking. In *4th International symposium on Gluten-Free Cereal Products and Beverages*.

- Van Spronsen, F. J., & Burgard, P. (2008). The truth of treating patients with phenylketonuria after childhood: the need for a new guideline. *Journal of inherited metabolic disease*, *31*(6), 673-679.

- Vittadini, E. & Y. Vodovotz (2003) Changes in the physicochemical properties of wheat- and soy-containing breads during storage as studied by thermal analyses. *Journal of Food Science*, 68, 2022-2027.

- Walser, M., & Hill, S. (1999). Can renal replacement be deferred by a supplemented very low protein diet?. *Journal of the American Society of Nephrology*, *10*(1), 110-116.

- Walstra, P. (2002). *Physical chemistry of foods*. CRC Press.

- Webster, A. C., Nagler, E. V., Morton, R. L., & Masson, P. (2017). Chronic kidney disease. *The Lancet*, *389*(10075), 1238-1252.

- Witczak, M., Juszczak, L., Ziobro, R., & Korus, J. (2012). Influence of modified starches on properties of gluten-free dough and bread. Part I: Rheological and thermal properties of gluten-free dough. *Food Hydrocolloids*, *28*(2), 353-360.

- Witczak, M., Ziobro, R., Juszczak, L., & Korus, J. (2016). Starch and starch derivatives in gluten-free systems–A review. *Journal of Cereal Science*, *67*, 46-57.

- Witczak, T., Juszczak, L., Ziobro, R., & Korus, J. (2017). Rheology of gluten-free dough and physical characteristics of bread with potato protein. *Journal of Food Process Engineering*, 40(3).

- Xu, W., Li, S., Whitely, N., & Pan, W. P. (2005). Fundamentals of TGA and SDT.

- Yazar, G., Duvarci, O., Tavman, S., & Kokini, J. L. (2017). Non-linear rheological behavior of gluten-free flour doughs and correlations of LAOS parameters with gluten-free bread properties. *Journal of Cereal Science*, 74, 28-36.

- Zannini, E., Kingston, W., Arendt, E. K., & Waters, D. M. (2013). Technological challenges and strategies for developing low-protein/protein-free cereal foods for specific dietary management. *Food research international*, *54*(1), 935-950.

- Ziobro, R., Juszczak, L., Witczak, M., & Korus, J. (2016). Non-gluten proteins as structure forming agents in gluten free bread. *Journal of food science and technology*, *53*(1), 571-580.

- Ziobro, R., Korus, J., Juszczak, L., & Witczak, T. (2013b). Influence of inulin on physical characteristics and staling rate of gluten-free bread. *Journal of Food Engineering*, 116(1), 21-27.

- Ziobro, R., Korus, J., Witczak, M., & Juszczak, L. (2012). Influence of modified starches on properties of gluten-free dough and bread. Part II: Quality and staling of gluten-free bread. *Food Hydrocolloids*, *29*(1), 68-74.

- Ziobro, R., Witczak, T., Juszczak, L., & Korus, J. (2013a). Supplementation of gluten-free bread with non-gluten proteins. Effect on dough rheological properties and bread characteristic. *Food Hydrocolloids*, *32*(2), 213-220.

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Chapter 2

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Study of gluten-free bread formulations based on rice and buckwheat flours: Part I

Abstract

In this first part of the work, the study on the effect of cellulose derivatives hydrocolloids on the rheological behavior of gluten-free bread formulations is presented. The aim of the study was to improve both the texture and the nutritional quality of the final product, increasing its fiber content without decreasing its technological quality. A whole-wheat flour bread recipe was used as a control. Wheat flour has been replaced with a mixture of rice and buckwheat flour and, to mimic the role of gluten, Hydroxypropyl methylcellulose (HPMC) and Carboxymethyl cellulose (CMC) were added to the formulation and used separately at four different percentages (0.1 %, 0.5 %, 1 %, 1.5 % on flour basis). The effect of water content on rheological properties of dough was investigated for samples prepared at 1% of hydrocolloids, considered the most promising formulations. It was observed that high water levels, decreasing the material consistency and modifying the structuring phenomena at high temperature, lead to doughs which are more similar to the benchmark. Positive results were also confirmed by the qualitative analysis of bread samples obtained by lab scale baking tests. Finally, it is worth noticing that proposed formulations, having a high dietary fiber content, are characterized by improved nutritional quality, in agreement with the current suggestions for healthy diet.

2.1 Introduction

The increasing interest for gluten-free (GF) products, constitutes a major challenge in relation to the development of healthier GF food with a high nutritional value and attracting organoleptic properties, mainly when baked products, such as bread, are considered.

The GF dough has a different consistency compared to the one made from wheat flour (WF). It also exhibits different properties as far as cohesion is concerned (Miyazaki & Morita, 2005; Onyango et al, 2009) and the resultant bread is usually characterized by defects in texture caused mainly by the presence of repulsive force among starch granules in the unstable suspension of starch, water and yeast. The GF dough is unable to entrap the air incorporated within the dough in the mixing phase and the carbon dioxide produced by yeast fermentation, this is due to a structure not coherent enough to allow the dough to rise. On one hand, some gases are released at an earlier stage, whereas, on the other hand, others remain trapped as they

go on to form unstable and irregular cells. The final product consists of a bread with a hard and irregular texture , as well as also being crumbly (Onyango et al, 2009).

GF bread is mostly poor in quality with a crumbling texture. It also tends to exhibit a dry, friable crumb, lacking in taste and colour as well as presenting further issues following the baking process (Gallagher et al, 2004; Onyango et al, 2009).

The main problem in producing GF bread is that gluten-free alternatives flours are unable to generate viscoelastic dough when mixed with water. Furthermore, GF bread also tends to include low dosages of proteins and dietary fibers. Even though some research has been carried out recently in relation to the ways in which dietary fiber can be used in GF formulations (Hager et al, 2011; Phimolsiripol et al, 2012; Sabanis et al, 2009), the limited amount of available data does not provide a substantial amount of support when attempting to explain the consequences of combining fibers to enhance the appearance, taste and smell of GF bread.

Many alternatives to wheat flour, including pseudocereals, even if generally considered of a lower quality in comparison to other types of crops, have been taken into account as safe and potentially beneficial to individuals' health as human foods. Among these pseudocereals, buckwheat is interesting for its high content of flavonoids and polyphenols and a high antioxidant activity that helps to reduce the risk of major chronic diseases (Costantini et al, 2014; Tian et al, 2002; Watanabe et al, 1997).

Because of its many beneficial properties, buckwheat also has the potential to prevent diabetes and improve levels of glucose tolerance in individuals who are already coping with the disease. This is mainly due to the fact that its carbohydrates are easily and slowly digested compared to other types (Mariotti et al, 2013); buckwheat is also interesting for its positive impact on lowering the level of serum cholesterol and for its high mineral content (Mariotti et al, 2013). Moreover, buckwheat is one of the best plant sources of proteins with high biological value. It is an interesting source of dietary fibre (Mariotti et al, 2013) and with a resistant starch content, which has beneficial effects through its ability to decrease the glycemic and insulin indexes (Skrabanja et al, 2001). An enrichment of gluten free mixtures with buckwheat, could therefore represent an interesting opportunity for GF formulations, even if not enough is known about its effects on the technological quality of the final product (Mariotti et al, 2013).

As well as numerous health benefits, the addition of dietary fibers could help in the changing and improvement of texture, sensory characteristics and shelf-life of foods (due to its water binding capacity, gel forming ability, fat mimetic, textural and thickening effects), even if most of the information on these aspects is related to studies on gluten-based food systems.

Further research is necessary to understand the effects of using or adding various fibers to GF dough systems.

In addition to buckwheat, rice flour (RF) is another suitable alternative for the preparation of GF products because of its many properties. RF is well accepted and one of the most used cereal grain flours for the production of GF products due to its bland taste, white colour, high digestibility, and hypoallergenic properties (Marco & Rosell, 2008). Furthermore, it is also low in protein, sodium, fat, and it contains a high percentage of easily digested carbohydrates. As far as nutritional quality is concerned, rice-based GF formulations have, in particular, low contents of vitamins, minerals, and dietary fiber (Phimolsiripol et al, 2012; Sciarini et al, 2010; Thompson et al, 2005). Consequently, the enrichment of GF rice bread with dietary fiber seems to be necessary. Due to its composition and protein content, rice flour does not have the capacity to build a viscoelastic network able to entrap gas cells, therefore gum or emulsifiers, as well as dairy products or enzymes, must be used in conjunction with rice flour, in order to obtain a suitable viscoelastic mixture that can mimic the role of gluten in providing structure and retaining gas (Gujral & Rosell, 2004).

Hydrocolloids in particular, may be essential for improving the texture and the appearance of the final GF product as the properties of certain hydrocolloids and mixture of hydrocolloids could mimic gluten (Mariotti et al, 2013) or provide other functions.
These agents create a cell network that is sufficiently strong to retain carbon dioxide during proofing, increasing loaf volume, andimproving dough cohesiveness by inducing attraction between the starch granules and possibly bind them thereby decreasing their mobility. Furthermore, these agents temporarily bind water required to gelatinize starch, structure the crumb and decrease crumb firmness (Gallagher et al, 2004).

In studies about GF bread based on rice flour, researchers used different gums, enzymes, and dietary fibers to develop GF bread formulations (Gallagher et al, 2004). Among hydrocolloids, cellulose and its modified forms are widely used in GF formulations, not providing significant nourishment or calories (BeMiller, 2007).

Cellulose derivatives are obtained by chemical modification of cellulose, which ensures their uniform properties. Among the different potential modifications, hydroxypropyl methylcellulose and carboxymethylcellulose seem particularly interesting. The behavior of hydroxypropyl methylcellulose (HPMC) was studied in addition to emulsifiers and it was demonstrated that HPMC forms a thermo-reversible gel on heating and builds interfacial films at the boundaries of the gas cells that confer some stability to the cells against the gas expansion and processing condition changes (Guarda et al, 2004). In fact it was observed (Marco & Rosell,

2008; Sabanis & Tzia, 2011) that HPMC is able to improve volume and texture of rice-based gluten free bread in terms of gas retention and water absorbing characteristics.

Carboxymethylcellulose (CMC) is also widely used for maintaining moisture and improving the mouth-feel and structural consistency of bakery products; it is also used in combination with other stabilizers and gums because of its high water-absorbing capacity (Mohammadi et al, 2014). CMC in GF formulations has the ability to improve specific volume, diminish crumb firmness, and decrease amylopectin retrogradation (Sciarini et al, 2012); moreover, GF formulation with CMC has received positive sensory evaluation by consumer panels (Lazaridou et al, 2007).

Although scientific research supporting GF formulations with HPMC or CMC is present, only a small number works include a rheological investigation to discuss some aspects of these products; therefore, a deeper rheological study of GF formulations with added hydrocolloids can be useful in improving these systems for potential commercial uses.

In the first part of this work, different formulation GF bread dough model, based on buckwheat, rice flour and cellulose derivatives hydrocolloids, are investigate from a rheological point of view. Particular attention is given to the in improvment of bread quality and texture and the enhancement of the fiber content and nutritional value of the final product without decreasing its technological quality.

2.2 Materials and Methods

2.2.1 Bread ingredients

A typical bread formulation was adopted to prepare a control dough (WF) with wholemeal wheat flour (Fior di Molino, Molino Rossetto, Italy), water (70%), salt (0.55%) and fats (9.7%). All components are given on weight flour basis. The fat used in this work is a structured water in oil emulsion (12% w/w water) (Lupi et al, 2011). Main flour components (i.e. protein, carbohydrate and fats), according to producer nutritional facts, are shown in table 2.1.

	WF (w/w%)	WBF (w/w%)	R (w/w%)
Total carbohydrate	61.6	70.1	75.4
Sugar	n.a.	0.6	0.7
Fat	1.5	2.1	1.3
Dietary fibre	7.1	7.5	1.6
Protein	11.2	11.2	6
Salt	n.a.	0.2	0.04

Table 2.1 Flour components.

Starting from this formulation, different gluten-free (GF) doughs were obtained by replacing the total amount of wholemeal flour with either rice flour (R) (Farina di Riso Finissima, "Nutrifree" NT Food Spa, Italy) or wholemeal buckwheat flour (WBF) (Farina di Grano Saraceno, "Nutrifree" NT Food Spa, Italy) or a mixture of the two gluten-free flours (mixture of WBF and R flour ratio 1:3). Some details on GF flours are shown in table 2.1. Afterwards, the effects of different hydrocolloids and different water contents (70%, 85%, 100% on flour basis) were tested. Two different cellulose derivatives hydrocolloids were used: hydroxypropyl methylcellulose (Acros Organics, USA) and Carboxymethyl cellulose (Acros Organics, USA). Hydrocolloids were added at four different percentages (0.1%, 0.5%, 1% and 1.5%) on total flour basis .

All doughs formulations are summarized in Table 2.2. Samples used in baking trial were prepared adding to the dough also commercial fresh yeast to dough (3.3% on flour basis) (Conad, Italy).

2.2.2 Samples preparation

All samples were prepared using the same procedure. Dry ingredients (i.e. flour, salt and hydrocolloids, if present) and fats were mixed together for 1 min using a N50 Hobart mixer (Hobart Corporation, USA), equipped with a dough hook, at speed 1 (over three positions) corresponding to 136 rpm of the agitator (according to the manufacturer information). Water was preheated at 40°C and then added to the premixed ingredients. After adding water, the dough was mixed for a further 15 min at speed 2 (corresponding to 281 rpm of agitator) to complete the mixing process. No yeast was added to prepare dough samples for rheological tests, in order to prevent any bubbles which could disturb the measurements. On the contrary, for doughs used in baking tests, yeast (3.3 % w/w) was dissolved in the preheated water and added to the mixture.

Sample ID	Wholemeal Flour (%w/w)	Rice Flour (%w/w)	Buckweath Flour (%w/w)	Water (%w/w)	CMC (%w/w)	HPMC (%w/w)	T_o (°C)	T_p (°C)
WF	100	-	-	70	-	-	67.8±0.9[a]	81±2[a]
R	-	100	-	70	-	-	50±1[b]	72.2±0.7[b]
WBF	-	-	100	70	-	-	65.1±0.1[a]	76±1[ad]
WBF-R	-	70	30	70	-	-	58±2[c]	73.0±0.7[b]
WBF-RH 0.1	-	70	30	70	-	0.1	45.2±0.6[d]	73.9±0.6[b]
WBF-RH 0.5	-	70	30	70	-	0.5	49±2[de]	73.7±0.4[b]
WBF-RH 1.0	-	70	30	70	-	1	49.4±0.4[e]	74.42±0.01[b]
WBF-RH 1.5	-	70	30	70	-	1.5	51.0±0.4[e]	75.47±0.01[c]
WBF-RH 85%W	-	70	30	85	-	1	56.58±0.02[c]	73.53±0.01[b]
WBF-RH 100%W		70	30	100	-	1	59±2[c]	74.1±0.3[b]
WBF-RC 0.1	-	70	30	70	0.1	-	59±1[c]	74.7±0.4[b]
WBF-RC 0.5	-	70	30	70	0.5	-	62±2[c]	76.9±0.6[d]
WBF-RC 1.0	-	70	30	70	1	-	59±1[c]	78±1[d]
WBF-RC 1.5	-	70	30	70	1.5	-	56±1[c]	76.9±0.6[d]
WBF-RC 85%W		70	30	85	1	-	57±3[c]	75.4±0.6[d]
WBF-RC 100%W		70	30	100	1	-	60.2±0.7[c]	75.3±0.3[d]

Table 2.2   1 Composition of investigated samples (concentrations based on wheat flour mass), onset gelatinization temperature (T_0), end temperature of the gelatinization process (T_p)

2.2.3 Rheological characterization

At the end of mixing, the dough was collected, wrapped in waxed paper, to reduce water loss, and left to stand at 4°C, for thirty minutes within an airtight box, to relax the stresses induced by the mixing process. Rheological tests were performed with a stress-controlled rheometer DSR 500 (Rheometric Scientific, USA) equipped with parallel plates of 25 mm and a Peltier temperature control device. The gap used during the tests was approximately 2.0±0.2 mm. Frequency sweep tests (at 30°C, 50° C and 70°C) were performed, increasing frequency from 0.1 Hz to 10 Hz, in the linear viscoelastic region, previously determined by stress sweep tests. Temperature ramp tests (time cure) in linear regime were also run at 1Hz, increasing temperature from 25°C to 90°C, at 1 ° C/min and modifying the applied stress in order to guarantee linear viscoelastic conditions over the whole temperature range. When running tests at high temperature, water loss was prevented by covering the sample rim with a thin layer of silicon oil (viscosity 1 Pa*s, VWR Chemicals, France).

Each sample was prepared twice and each test was carried out in triplicate, all data are shown in terms of mean value and standard deviation.

From a rheological point of view, dough is a very complex system, often described as a solid-like material that behaves as a "weak gel" in the frequency range usually investigated (0.1-100 Hz) (Gabriele et al, 2001; Migliori & Gabriele, 2010; Ng & McKinley, 2008; Peressini et al, 2002).

According to the "weak gel model" the material can be described, in a schematic way, as a three-dimensional network formed by interacting rheological units; the experimental linear behavior of dynamic moduli vs frequency in a double log plot can be described by fitting the complex modulus, i.e. the combination of G' and G", with a two parameters power law (Gabriele et al, 2001)

$$G^*(\omega) = \sqrt{(G')^2 + (G'')^2} = A \cdot \omega^{\frac{1}{z}} \quad (2.1)$$

where A is related to the strength of the three-dimensional network and z is a measure of the network extension.

2.2.4 Baking tests and physical characteristics

For samples used in baking tests, at the end of the mixing process, the dough was placed into a baking tin, covered with foil and placed in a proofer at 40°C for 2 h. After proofing, loaves were baked in a fan oven (LineMiss XFT113, Unox, Italy) for 50 min at 140°C. The oven was preheated at 140°C for 5 min. All baked products were cooled down and analyzed after having reached room temperature. Color appearance is a key quality indicator for many baked goods, therefore colorimetric measurements were carried out on crust after baking, with a Minolta Colorimeter (Chroma-Meter CR-400, Konica Minolta Sensing, Osaka, Japan). Using the CIELab color system the parameters obtained were : L* (lightness), a* (-green, + red), b* (-blue, +yellow) (Costantini et al, 2014). They were obtained as average of 4 different determinations in different points of sample (either crust or crumb). For the purpose of qualitative comparison, images of whole bread and of internal crumb were taken for selected samples.

2.2.5 Lubricated squeeze flow

During processing, doughs are subjected to large deformations. The average deformation rates of the dough vary greatly: from 10^{-3} s^{-1} during proofing or oven rise, up to 100 s^{-1} during mechanical dough development (Bloksma, 1990). Consequences of large deformations on breadmaking performance can be investigated studying the extensional viscosities determined by lubricated squeezing flow tests.

Biaxial extension tests were performed on cylindrical dough samples according to Kokelaar and coworker (Kokelaar et al., 1996). Compression tests were performed with a Zwick material testing machine equipped with a 5±0.02 kN load cell. The cylindrical doughs were compressed uniaxially between two plates with the same initial diameter (R_0) as the test piece (36 mm) at a constant speed, and care is taken to minimize friction between the tested material and the plates by lubrication with paraffin oil. The force, *F(t)*, is recorded continuously as a function of time. Through lubricated tests, the biaxial stress σ_B [Pa], is determined from the compression force *F(t):*

$$\sigma_B = \frac{F(t) \cdot h(t)}{\pi R_0^2 \cdot h_0} \quad (2.2)$$

Where h(t) is the instantaneous height separating the plates [m], and h_0 is the initial sample thickness (m). Assuming that the deformation was uniform and the material was

incompressible, equibiaxial strain ε_B can be calculated according to (Soskey and Winter, 1985; Takahashi et al., 1993)

$$\varepsilon_B = -\frac{1}{2}\left(\frac{h_0 - \delta}{h_0}\right) (2.3)$$

where δ is the displacement $\delta = u_z t$, were u_z, is the upper plate downward speed [m/s] and t is the time [s]. The biaxial extensional strain rate $\dot{\varepsilon}_B$ (s^{-1}) is therefore defined as:

$$\dot{\varepsilon}_B = \frac{d\varepsilon_B}{dt} (2.4)$$

$$\dot{\varepsilon}_B = \frac{1}{2}\left(\frac{u_z}{h_0 - u_z t}\right) (2.5)$$

The initial height of the test of the samples was 14±1 mm. The deformation force was recorded at three different compression speeds (0.1 mm/s, 0.3 mm/s, 0.5 mm/s) at room temperature, and the biaxial extensional viscosity was calculated from the following relation:

$$\eta_B = \frac{\sigma_B}{\dot{\varepsilon}_B} \ (2.6)$$

2.2.6 Statistical analysis

All data are shown in terms of mean and standard deviation. A statistical analysis, when necessary, was carried out using one-way ANOVA and t-student test (MS Excel 2013, Microsoft, USA) and differences among compared samples were considered significant at p-value <0.05 (interval of confidence of 95%).

2.3. Results and discussion

2.3.1 Effects of different flours

The evolution of rheological properties with temperature is depicted, for WF sample, in Fig. 2.1 in terms of storage and loss modulus and phase angle. It can be seen that, with increasing temperature, an initial decrease in both moduli is followed by a sharp rise that corresponds to the onset of starch gelatinization and it is due to the swelling of starch which tends to occupy the entire space available in the system and, eventually, form a 3D network (Baldino et al, 2014; Jekle et al, 2016; Migliori & Gabriele, 2010; Migliori et al, 2011).
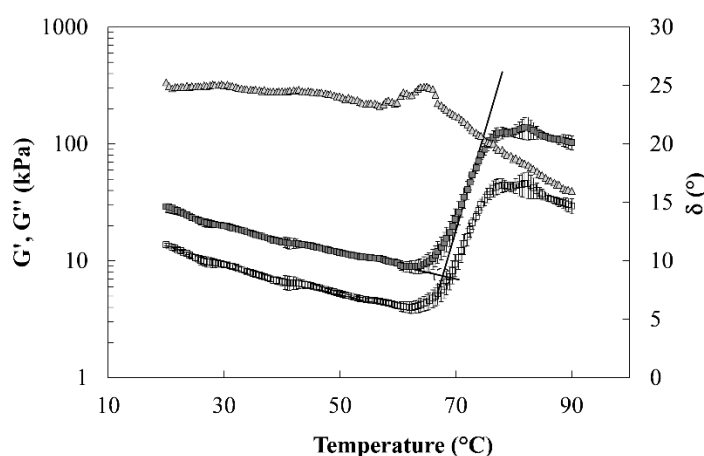
Figure 2.1 Storage modulus (G') , loss modulus (G'') and phase angle (δ) vs temperature for WF sample

The structure evolution is evidenced also by phase angle that is almost constant at low temperature, suggesting the lack of any modification in material microstructure, and significantly decreases at higher temperatures confirming the formation of a more structured network.

The completion of the gelatinization phenomena is evidenced by the peak in moduli; after this, any increase in temperature modifies the molecular mobility (Champenois et al, 1998; Migliori & Gabriele, 2010) or causes a disruption of granules and the melting of the crystalline structure (Champenois et al, 1998; Jekle et al, 2016) yielding a reduction in dynamic moduli. It is worth noticing that, even after the peak in moduli, phase angle still decreases suggesting a continous evolution in microstructure.

With the aim of determining, in a more quantitative way, the critical temperatures, corresponding to the gelatinisation phenomena, the G' trend was analysed in more in detail because the storage modulus is related to the solid-like behaviour and, therefore, it seems more related to the effect of the structure development. According to a procedure already proposed

in the literature for different starch based systems (Marino et al, 2014) a local linearization was performed in the first decreasing region and in the second increasing region considering the tangent to the curve inflection point (see Fig. 2.1). The onset of gelatinisation (T_o) was assumed as the intersection of the obtained straight lines and, as it can be seen in Fig. 2.1, it represents a good estimate of the knee point of G' curve.

The completion of gelatinization, related to peak in moduli, was assumed as the temperature corresponding to the maximum G' value (T_p).

When samples based on different flours are considered, a similar temperature dependence is observed (Fig. 2.2) even if differences in moduli values and critical temperatures are observed. It is worth noticing that for few samples (e.g R and WBF-R) transitions were more smoothed, with respect to WF, and the knee of the curve was less evident; nevertheless, T_o was estimated according to the procedure previously described because also in these cases it seems a good quantification of a critical temperature where the swelling phenomenon becomes evident enough to yield a relevant increase in G', even if with a slower rate.
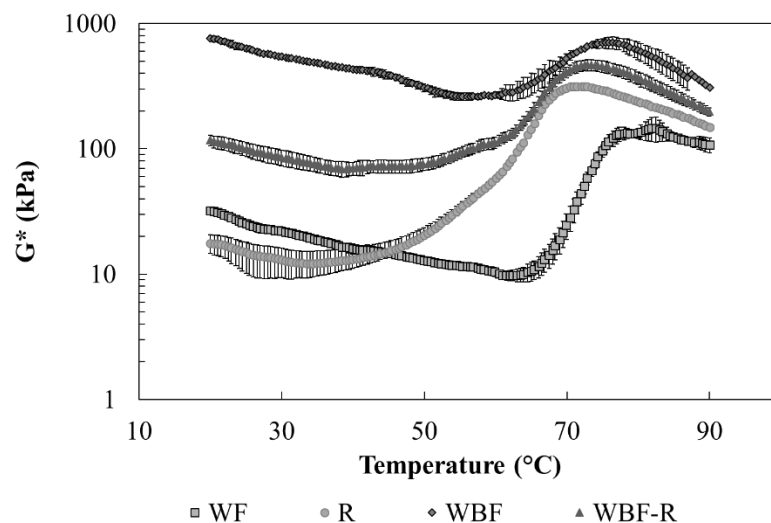
Figure 2.2 Complex modulus (G) vs temperature for WF, R, WBF, WBF-R*

Differences among tested samples, in T_o and T_p, can be mainly ascribed to both the different starch source and the different protein content. In fact, it is known that starch from different botanical sources exhibits different gelatinization properties (Ai & Jane, 2015; Wu et al, 2016). Moreover proteins negatively affect the network formation because they reduce the contact between the starch granules and delay the gelatinization, owing to protein-carbohydrate interactions (Champenois et al, 1998; Jekle et al, 2016). Protein can build a sort of "barrier", surrounding starch and limiting water diffusion to starch granules and, therefore, hindering the

gelatinization. This effect is not related to gluten only and it is observed in other starch-protein systems (Jekle et al, 2016).

Therefore, samples with a lower protein content (R and WBF-R) exhibit T_o values lower than those exhibited by dough with higher protein content (WF and WBF).

When systems with similar protein amount (i.e. WF and WBF) are considered, no statistical difference can be observed.

Finally, sample R is characterized by the lowest T_o value, probably owing to the low content of both fiber and protein that do not hinder gelatinization phenomena.

It is worth noticing that T_p follows the same trend exhibited by T_o, i.e. WF>WBF>WBF-R≅R (see table 2.2) confirming a delay, of gelatinization completion, in systems having a higher protein content.

Further details on rheological behavior of considered samples were obtained from frequency sweep tests performed at different temperatures.

Frequency sweep data at 25°C are shown, as an example, in Fig. 2.3 in terms of complex modulus and loss tangent, for all samples.
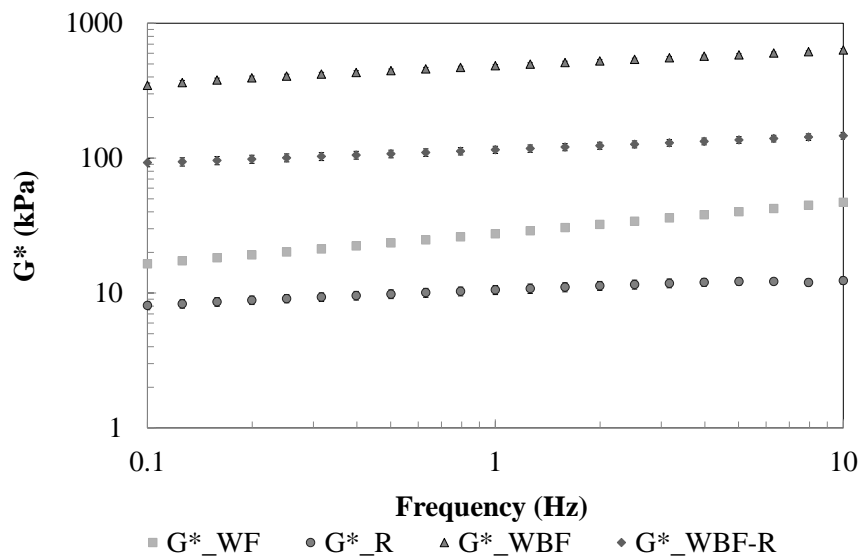
Figure 2.3a Frequency sweep test for WF, R, WBF, WBF-R sample, at 25°C
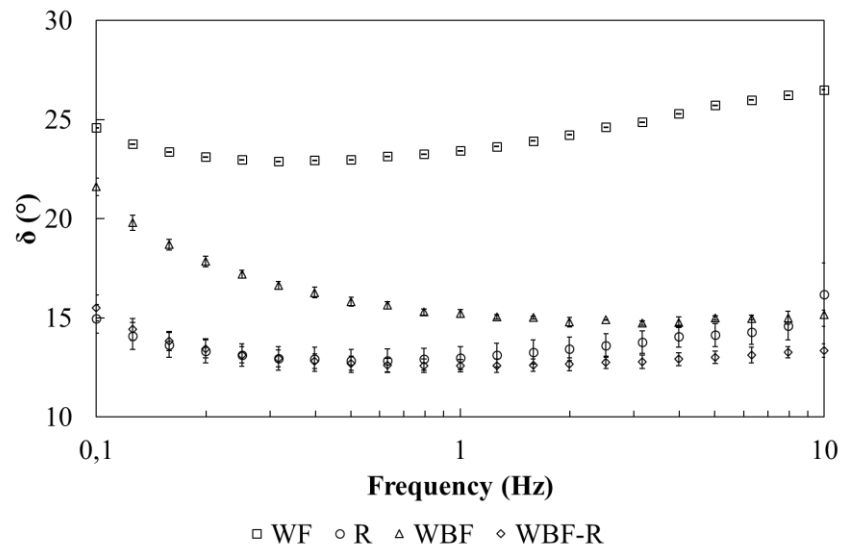
Figure 2.3b Phase angle (δ) trend in the investigated frequency range for WF, R, WBF, WBF-R sample, at 25°C

As expected, in a double log scale, complex modulus as frequency function exhibits a linear trend, within the considered frequency range, confirming a "weak gel" behavior of these systems. Moreover, for all samples, phase angle is lower than 45° (i.e. loss tangent is lower than unity) evidencing that G' is greater than G" and, therefore, they exhibit a prevalent solid-like behavior (Barnes et al, 1989).

Samples containing whole buckwheat flour (i.e. WBF and WBF-R) are characterized by the highest values of complex modulus (Fig. 2.3a); a similar result was obtained by Wu et al (2016), which investigated systems based on buckwheat and wheat flours. They attributed the observed differences to a potential high amylose content of buckwheat flour with respect to the wheat one. In a similar way it can be speculated that low values of R complex modulus can be attributed to the lower mean amylose content of rice starch with respect to wheat one (Singh et al, 2003).

As far as phase angle is concerned (Fig. 2.3b), it can be noted that samples based on rice flour exhibit the lowest values, suggesting a more relevant solid-like behavior; on the other hand, WF dough is characterized by high values (for example δ(1 Hz)=23.4±0.4) in good agreement with literature values for other common wheat doughs (see for instance (Migliori & Gabriele, 2010)). It is worth noticing that protein and starch are thermodynamically different polymers and therefore, their simultaneous presence can yield different results depending on the potential compatibility (Rao, 1999). In pure starch systems, starch granules are the rheological units building the network and controlling the rheological properties of the system. When gluten is present, it causes changes in the interactions among starch granules, modifying the network that is built, in this new condition, by cells made by protein strands filled by starch

(Rao, 1999). As a consequence, it can be speculated that protein presence weakens the network extension and leads to a material with a lower solid-like component, evidenced by the higher phase angle. This is in agreement with literature data, evidencing an increase in loss tangent at room temperature when gluten partially replaces pure starch (Jekle et al, 2016), and explains the relevant differences between WF and R flours observed in Fig. 2.3b.

Similar effects, even if less relevant, can be observed for WBF flour that has phase angle values intermediate between those of R and WF doughs; as already discussed, the observed behavior depends on the level of potential compatibility (or incompatibility) between starch and protein and on the nature of starch.

Frequency sweep data, at different temperatures and for all samples, were fitted with Eq. 1 and computed parameters (i.e. A and z) are reported in Fig.2.4.
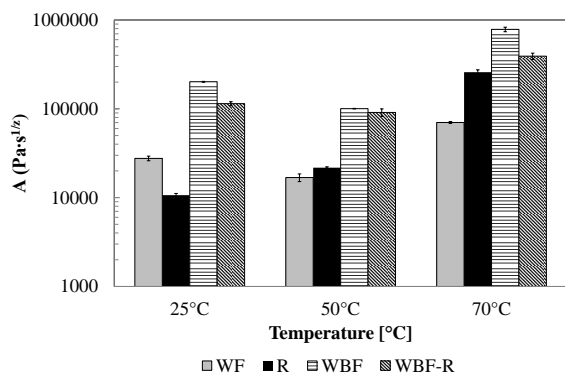
Figure 2.4a Parameter A (Eq.2.1) of the weak gel model for WF,R, WBF-R
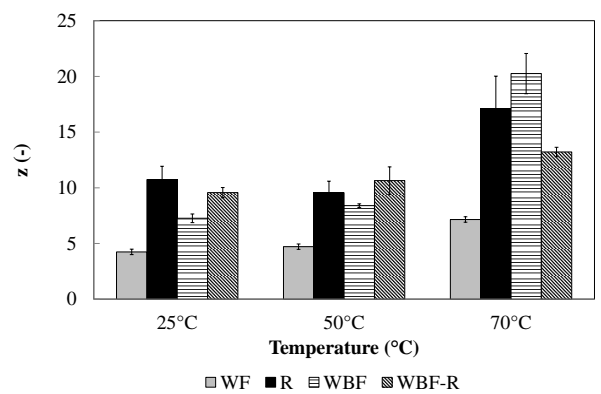
Figure 2.4a Parameter Z (Eq.2.1) of the weak gel model for WF,R, WBF-R

It can be seen that at 25°C, according to the previous discussion on G* and phase angle, whole meal buckwheat flour yields the most consistent samples, as evidenced by the large value of network strength (i.e. A, see Fig. 2.4a); on the other hand, the network extension, z, is very low for WF where gluten, probably, hinders starch-starch interactions, and is very high for R where protein content is lower (Fig. 2.4b). When temperature increases up to 50°C, the parameter A decreases for all samples, owing to the increased mobility, except for R where a slight increase is observed, owing to the starch swelling (onset of gelatinization for R flour was estimated equal to 50±1 °C, table 2.2); on the other hand no relevant change in z is observed, in agreement with phase angle trend during temperature ramp test.

A further temperature increase, up to 70°C, leads to the starch gelatinization evidenced by the relevant increase in both A and z. The effect is less evident for WF, owing to the high T_o value (close to 70°C, see table 2.2).

2.3.2 Effect of Hydrocolloids concentration

Among the investigated formulations, sample WBF-R was chosen as a promising starting point for hydrocolloid addition; in fact this sample has a high content in dietary fiber, responding to the recommendation of a higher daily intake of fibers. Moreover, among all formulations, it exhibits rheological properties not so different with respect to WF, in terms of both complex modulus and phase angle at different temperatures. Therefore hydrocolloid addition could be helpful in reducing the differences with WF yielding, finally, a potential GF bread with high fiber content.

Effects of HPMC addition

Figure 2.5 shows the behavior of samples added with HPMC in a temperature ramp test, in terms of complex modulus and phase angle.
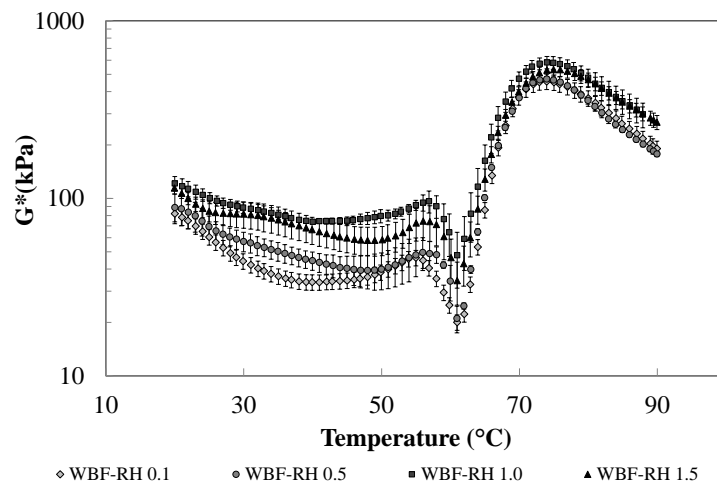
Figure 2.5a Complex modulus (G*) vs temperature for WBF-RH0.1, WBF-RH0.5, WBF-RH1, WBF-RH1.5
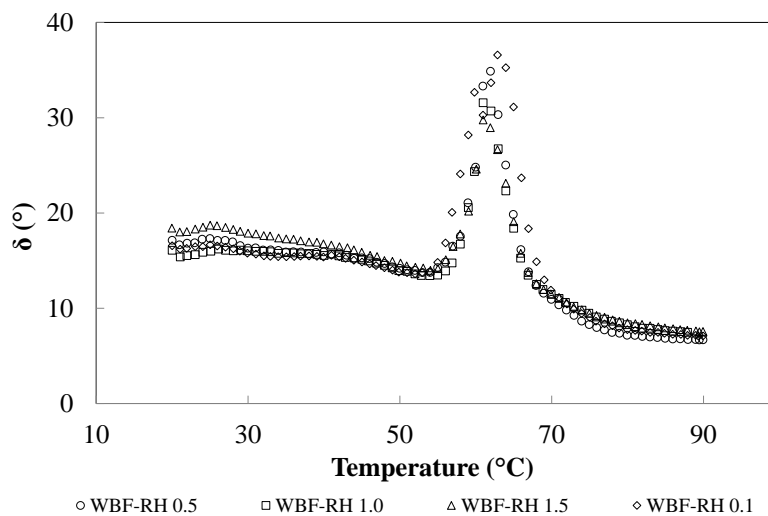
Figure 2.5b Phase angle (δ) trend vs temperature for WBF-RH0.1, WBF-RH0.5, WBF-RH1, WBF-RH1.5

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For all samples, an initial decrease in complex modulus is observed, due to kinetic effects; a further increase in temperature yields to a slight G* increase, owing to the onset of starch gelatinisation. After a small peak in G*, occurring at temperatures unexpectedly low, a sharp drop is then observed, leading to a minimum at 60.9±0.6°C (table 2.3).

Sample ID	T_{min} (°C)
WBF-RH 0.1	60.9±0.6 [a]
WBF-RH 0.5	61.2±0.4 [a]
WBF-RH 1.0	60.96±0.01 [a]
WBF-RH 1.5	60.97±0.02 [a]

Table 2.3 Minimum temperature (T_{min}) observed in the trend of the complex modulus G vs temperature*

Subsequently, G* increases up to a second peak and finally slightly decreases until the end of the test.

It is worth noticing that the narrow region, including the minimum in G*, corresponds to an evident peak in phase angle (Fig. 5b) that suggests a "temporary" drastic reduction in the solid-like behavior of the system.

This unexpected temperature behavior, which significantly differs from that observed for WBF-R dough, can be attributed to HPMC effects. In fact, the same rheological trend was observed during a temperature ramp test of a HPMC aqueous solution (Fairclough et al, 2012) and this was due to a phase separation (among a polymer-rich phase, a polymer-poor phase and an aqueous phase) preceding the HPMC gelation, where moduli increase (and phase angle decreases) because of the formation of a polymer network. It is worth noticing that the minimum in G', and therefore G*, observed in this work, is independent of the HPMC amount (no statistical difference, see table 2.3) and is the same found by (Fairclough et al, 2012), i.e. approximately 61°C, confirming that the same phenomenon was observed.

Starting from these considerations, it can be speculated that in the present systems the effects of starch gelatinisation are hidden, partially, by HPMC phase separation, making less evident the onset of swelling. After phase separation, the HPMC gelation and starch gelatinisation overlap each other yielding, as a final effect, a relevant peak in moduli. When gelation phenomena are completed, a further increase in temperature leads to the observed final slight decrease.

It is worth noticing that T_o and T_p, in the present case, are no longer representative of starch gelatinisation alone because, in the same temperature range, HPMC gelation and phase separation occurr. Nevertheless, they were computed also for samples containing hydrocolloids because they can be used to estimate the onset and the completion of structuring phenomena associated with relevant changes in dough thermal behavior.

An increase in hydrocolloid concentration does not affect the trend and the values of G^* for the different mixtures, but it has more influence on the consistency and then on the values of the complex modulus. Differences are evident mainly at temperatures lower than G^* minimum and it can be seen that the complex modulus increases with HPMC addition up to 1%; on the other hand, a further addition, up to 1.5%, yields a slight reduction in moduli. When T_o is considered, it can be seen that the structuring phenomena are shifted towards the higher temperature, when increasing HPMC, probably due to to the competition for water availability between starch and HPMC. This effect is not evident for T_p where statistical differences are observed only at the highest amount (see Table 2.2).

Frequency sweep tests were characterized by a linear trend, in a log-log plot, of dynamic moduli as frequency function (data not shown) and therefore, the weak gel model (Eq. 1) was applied obtaining the values of A and z reported in Fig. 2.6.
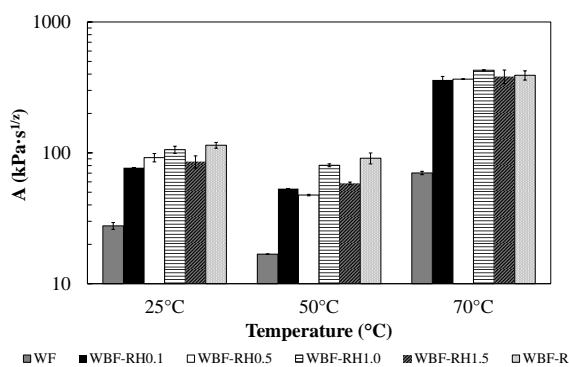
Fig. 2.6a Parameter A (Eq.2.1) of the weak gel model for WF, WBF-R, WBF-RH0.1, WBF-RH0.5, WBF-RH1, WBF-RH1.5
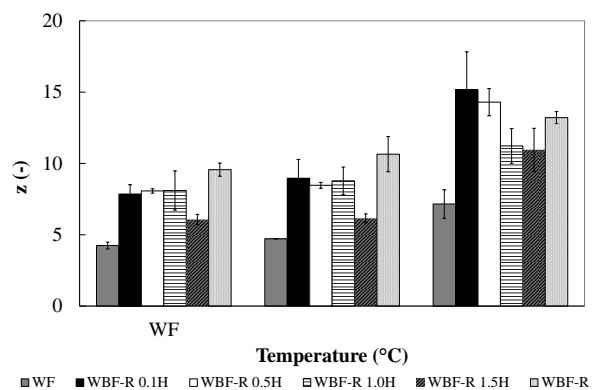
Fig.2.6b Parameter Z (Eq.2.1) of the weak gel model for WF, WBF-R, WBF-RH0.1, WBF-RH0.5, WBF-RH1, WBF-RH1.5

At 25°C, for all samples, except for WBF-RH 1.5, parameter A (Fig. 2.6a) increases with HPMC concentration and z (Fig. 2.6b) is almost constant; all values are slightly lower than those of WBF-R dough. It could be speculated that, due o the high water-binding capacity of this hydrocolloid (Sabanis & Tzia, 2011) which competes with starch for water, the formation of the initial starch-protein network is hindered; on the other hand HPMC, probably acts also

as a sort of filler, increasing the "consistency" of the material. The observed result is a compromise between these effects and, therefore, an initial increase in A is followed by a decrease at high concentration when, probably, the weakening effect on starch protein-network is more relevant than the "filler" effect.

When temperature is increased up to 50°C, A decreases and z increases, in agreement with the previous discussion on temperature ramp test, even if these changes are minimal. On the other hand, a further increase in T leads to relevant changes in both parameters owing to structuring phenomena involving both starch and HPMC. It is worth noticing that the value of A is almost the same for all samples, independently of HPMC content; on the contrary, z is almost constant for low hydrocolloid addition, i.e. 0.1% and 0.5% (and it is slightly higher than the value observed for WBF-R), whereas it decreases at higher concentration, suggesting the existence of an "optimal" HPMC concentration. It could therefore be assumed that, at low concentration, HPMC gelation and starch gelatinization have a synergetic effect, yielding a more structured network; at higher concentration the hydrocolloid hinders starch swelling because of its water-binding ability and therefore reduces the final network extension.

All doughs prepared with HPMC show values of A and z higher than those of standard dough; therefore, they tend to be more consistent, compact and less workable, evidencing that HPMC was not able to modify in a relevant way the starting material behavior.

Effects of CMC addition

All samples prepared with CMC exhibit a thermal behavior very similar to that of unmodified dough (Fig.2.7)
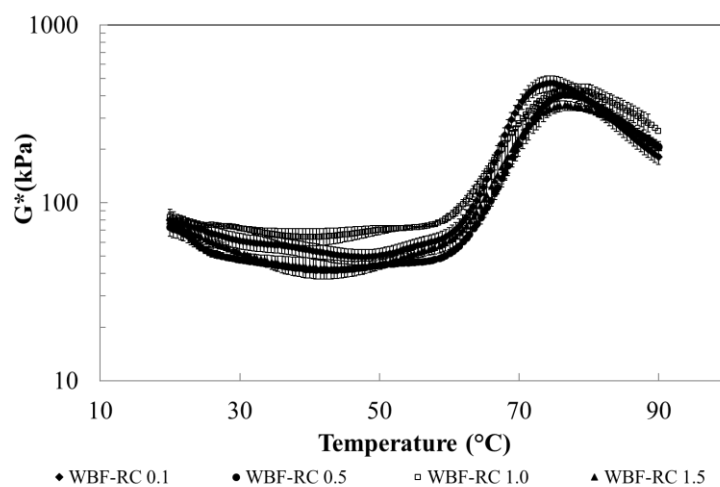
Fig. 2.7 Complex modulus (G*) vs temperature for WBF-RC0.1, WBF-RC0.5, WBF-RC1, WBF-RC1.5

With increasing temperature, the initial decrease in G* is followed by a more or less sharp rise and, after a peak, G* decreases until the end of the test. This trend is in agreement with literature data on gelation of CMC aqueous solutions (Fairclough et al, 2012) evidencing the lack of critical regions similar to those observed for HPMC. Therefore, starch gelatinization and CMC gelation overlap each other without modifying the typical trend, even if differences in moduli are present, as more clearly evidenced by the analysis of frequency sweep tests in terms of A and z. This is also confirmed by T_o values (Table 2.2) that do not show statistical difference with respect to that of WBF-R; on the other hand T_p increases when adding CMC, with respect to WBF-R, probably because of the CMC gelation, in fact for pure CMC aqueous solution a continuous increase up to 90 °C (final temperature of the test) has been observed by (Fairclough et al, 2012). Moreover, no statistical difference is observed among samples with different CMC concentrations. At 25°C all samples exhibit values of both A and z lower than those of WBF-R (Fig. 2.8); moreover, as already observed for HPMC addition, A increases with CMC fraction up to 1% and then decreases, whereas z decreases monotonously.
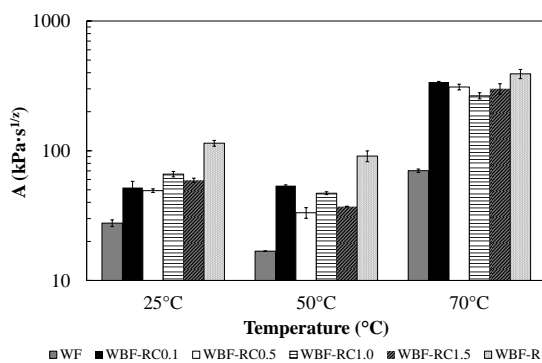
Fig.2.8a *Parameter A (Eq.2.1) of the weak gel model for WF, WBF-R, WBF-RC0.1, WBF-RC0.5, WBF-RC1, WBF-RC1.5*
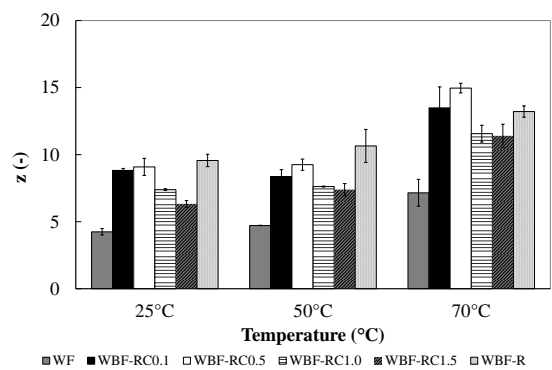
Fig.2.8b *Parameter Z (Eq.2.1) of the weak gel model for WF, WBF-R, WBF-RC0.1, WBF-RC0.5, WBF-RC1, WBF-RC1.5*

Data at 50°C shows a decrease in A, mainly due to kinetic effects; however differences are present because of the overlapping of CMC and starch behavior. Finally, at 70°C, structuring effects related to both starch and CMC are evident and lead to A values quite close to each other (slight differences are probably due to differences in gelation rate cause by the competition between gelling agents). Also in this case the network extension, for samples with low CMC fraction, is higher than that computed for unmodified sample, as already observed for dough containing HPMC.

2.3.3 Effect of water content

Among the investigated samples it seems that the most promising ones, as potential bases for gluten-free bread, are doughs prepared with higher hydrocolloid addition, i.e. 1% and 1.5%. It was observed that the use of CMC and HPMC is not able to modify in a relevant way the values of parameter A, i.e. G* at 1 Hz, and therefore the consistency; on the the other hand, a reduction in z, towards values closer to WF ones, was observed for both concentrations. The effects seem more pronounced at 1.5%; nevertheless differences with samples at 1% are quite small and with the aim of reducing the amount of additives (also for economic reasons), samples with 1% of additives were chosen to investigate the effects of different levels, increasing water from 70% (used for all samples) to 85% and then to 100%. All percentages refer to the total amount of flour, as reported in table 2.2.

Thermal behavior of HPMC added samples is shown in Fig. 2.9, a relevant reduction of the complex modulus is observed when increasing water content, as expected due to the dilution effect more evident when going from 70% to 85%. Moreover temperature dependence of G* is different because the increased amount of available water modifies the gelatinisation phenomena.
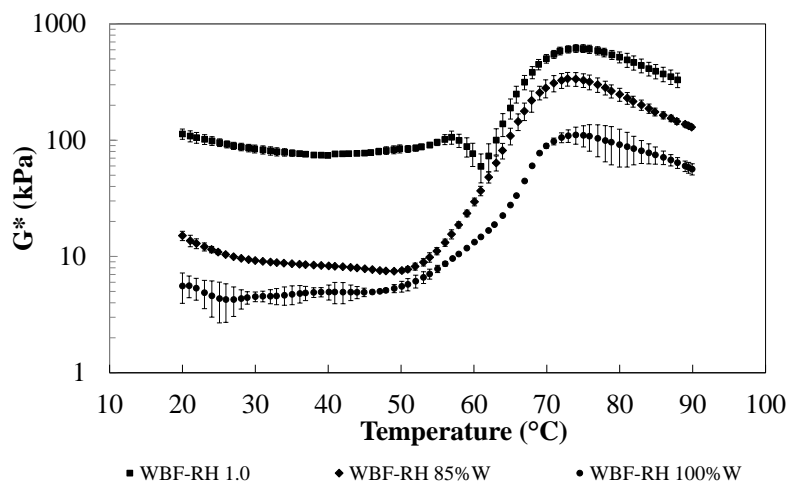
Fig. 2.9 Complex modulus (G*) vs temperature for WBF-RH1.0, WBF-RH85%W, WBF-RH100%W

It is worth noticing that the minimum in G*, caused by the phase separation of HPMC, disappears at higher water content. This can be attributed to an extended starch swelling and gelatinisation, caused by the higher water amount, that can balance the drop in moduli, making it not visible from a macroscopic point of view. It is worth noticing that differences in G* decrease with increasing temperature due to the structuring phenomena (of both starch and HPMC) promoted by the larger amount of available water.

These effecst are more evident in A and z parameters (Fig. 2.10); it can be seen that differences in A decreases at higher temperature whereas z, related to network extension, increases with increas

ing water.
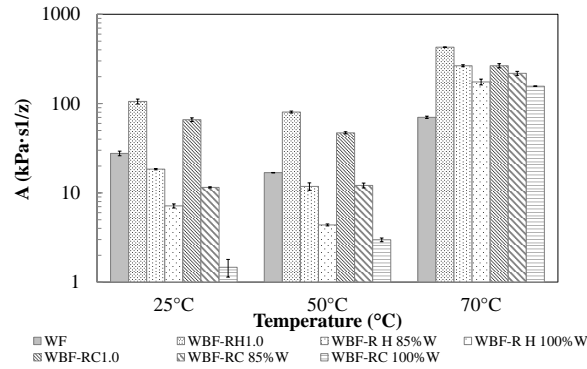
Fig. 2.10a Parameter A (Eq.2.1) of the weak gel model for samples prepared with 1% of hydrocolloid (HPMC and CMC) and at different water concentration
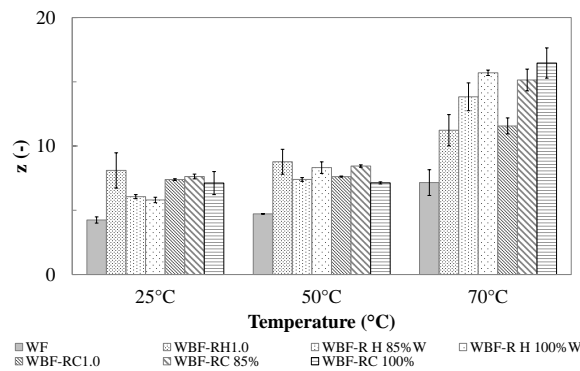
Fig. 2.10b Parameter Z (Eq.2.1) of the weak gel model for samples prepared with 1% of hydrocolloid (HPMC and CMC) and at different water concentration

A similar behavior was observed also for samples added with CMC (see Fig.2.11): complex modulus decreases significantly at room temperature, with increasing water content, and differences almost disappear at high temperature due to gelation phenomena which leads, also, to the network extension as confirmed by weak gel model parameters (Fig. 2.10).
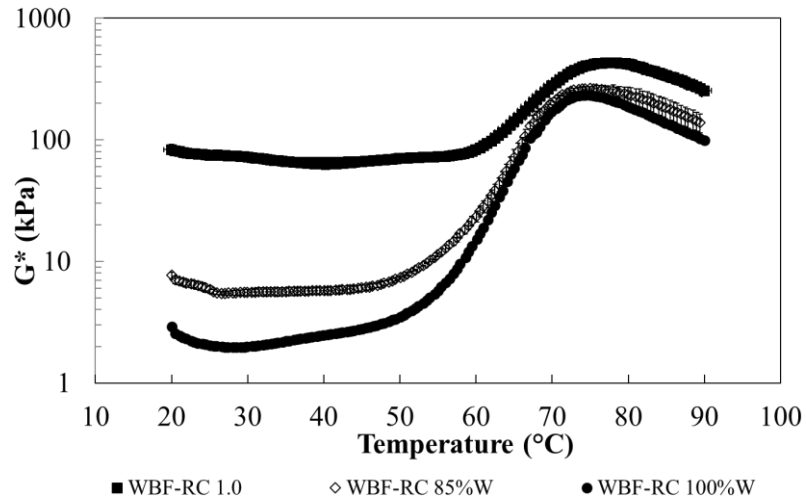
Fig.2.11 Complex modulus (G*) vs temperature for WBF-RC1.0, WBF-RC85%W, WBF-RC100%W

It is worth noticing that samples with high water content exhibit an interesting behavior, even if differences with respect to the benchmark (i.e. WF) are still relevant: water increase leads to a reduction in moduli, at low T, that makes these systems more workable and more similar to WF, even though obtained values are lower than desirable (lower than values observed for WF). At higher T, differences with respect to the benchmark decrease and a similar thermal behavior is observed. It could be speculated that a "softer" material at room temperature could ease the process of the gas cell expansion (during the leaving stage) and the obtained cell structure could be consolidated by structuring phenomena at high temperature which occur in a range close to that of WF and with similar moduli values (as suggested by weak gel parameters). It seems that for CMC the best compromise could be obtained with sample WBF-RC 85%W, where reduction at low T is not very drastic (as observed for WBF-RC 100%W) whereas for HPMC, a good proposal could be WBF-RH 100%W where moduli at high T are closer to those of WF (and effects of diluition at room temperature are not so relevant as for CMC).

2.3.4 Baking test results

Results of colorimetric analysis of baked products are shown in table 2.4, in terms of CIELab paramters measured for both crust and crumb.

When samples without hydrocolloids are considered, it can be seen that the R bread is very different with respect to the WF, mainly in terms of lightness (for both crust and crumb), a* (for crust) and b* (for crumb). As expected, the general result for R flour is a lighter bread with respect to a standard wheat bread. When wholemeal buckwheat flour is used, differences are still present, but they are less evident (Table 2.4). The use of both hydrocolloids seems to yield a slight increase in L*, with increasing concentration, whereas no clear trends are present in a* and b*. Finally, the change in the water level does not lead to relevant differences in colorimetric parameters.

The visual inspection of samples WF, R, WBF and WBF-R (Fig. 2.12) confirms the discussed differences in colorimetric parameters; moreover, it highlights differences in loaf volume and crumb porosity of GF samples with respect to the benchmark.

| | | | |
| WF | R | WBF | WBF-R |
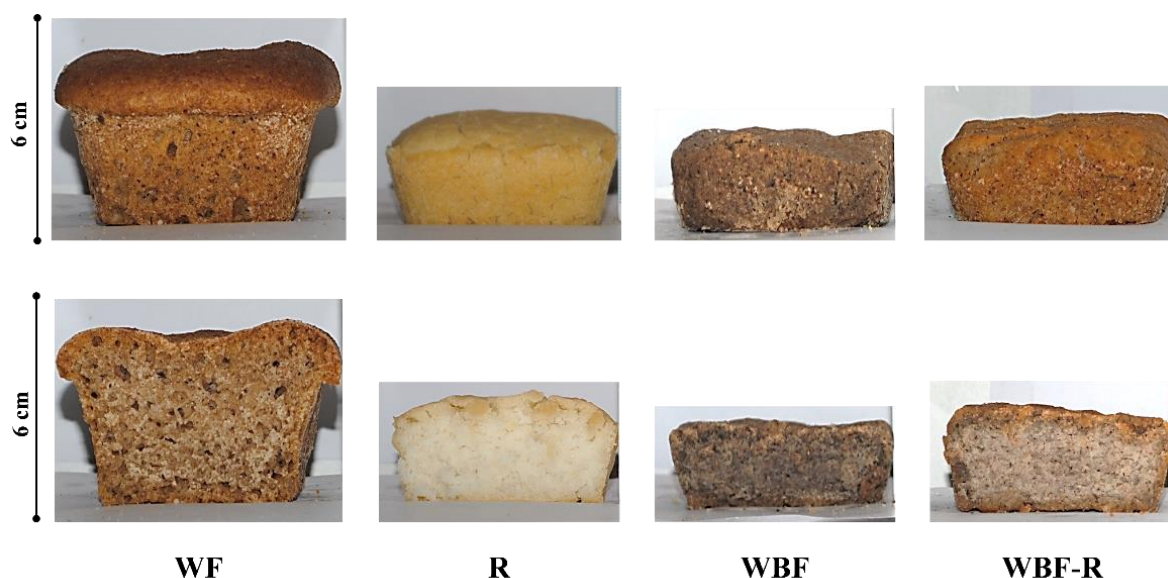
Fig. 2.12 Bread loaf height and internal structure of the GF control samples (WF, R, WBF, WBF-R)

Even this qualitative inspection seems to suggest that, among tested materials, WBF-R is the most suitable for further modifications. Images of internal crumb were taken also for samples added with 1% hydrocolloids at different water levels (Fig. 2.13),
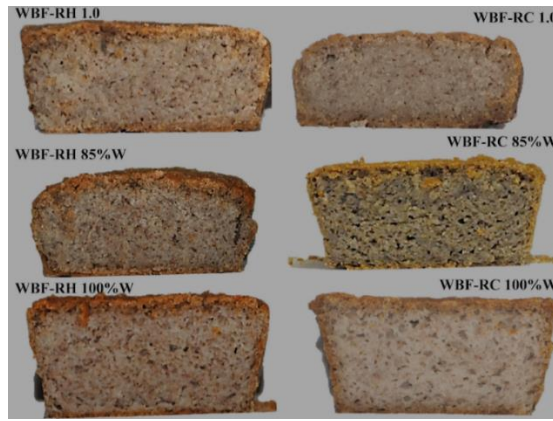
Fig. 2.13 Internal structure of the GF bread prepared with 1% of hydrocolloid and using different water content

From Fig. 2.13, it can be seen that high water fractions lead to a more porous structure qualitatively closer to the benchmark. It seems that the best results (i.e. a crumb more similar to that of WF) are obtained for WBF-RC 85%W, WBF-RH 85%W and WBF-RH 100%W, in agreement with the previous discussion on rheological properties.

Sample ID	Crumb			Crust		
	L* (-)	a* (-)	b* (-)	L* (-)	a* (-)	b* (-)
WF	52±1	3.7±0.8	18±1	47±1	11.5±0.6	28.9±0.8
R	78±3	2.3±0.3	11.8±0.8	70±1	0.105±0.001	28.72±0.08
WBF	53±1	3.9±0.3	11±1	45.9±0.6	7±1	22±1
WBF-R	61±2	2.5±0.4	12±1	53±1	10.7±0.7	33.5±0.4
WBF-RH 0.1	59±1	2.6±0.2	11.7±0.5	47.8±0.5	9.7±0.2	30.5±0.8
WBF-RH 0.5	61±0.9	2.8±0.5	13±2	51.2±0.5	11.3±0.4	33±1
WBF-RH 1.0	63±1	2.1±0.3	11±1	54.5±0.5	10.5±0.5	33±1
WBF-RH 1.5	65±1	2.1±0.3	12±1	53.2±0.4	8.95±0.05	28.8±0.1
WBF-RH 85%W	64±1	2.3±0.3	12±1	47.8±0.5	9.0±0.4	29.1±0.1
WBF-RH 100%W	63±1	1.9±0.1	10±2	52.3±0.3	8.9±0.2	31.6±0.8
WBF-RC 0.1	59±2	2.9±0.2	12.3±0.7	47.8±0.5	13.6±0.1	33.0±0.2
WBF-RC 0.5	58±1	2.9±0.2	12.2±0.8	48.6±0.4	13.3±0.2	33.4±0.1
WBF-RC 1.0	59±2	3.0±0.2	12±1	47.7±0.5	11.9±0.5	32.3±0.3
WBF-RC 1.5	60±2	2.8±0.2	12.2±0.7	49.7±0.9	12.3±0.4	34.2±0.7
WBF-RC 85%W	61±1	2.2±0.2	11.2±0.6	47.5±0.5	12.5±0.4	33.2±0.4
WBF-RC 100%W	61±1	2.9±0.5	11.3±0.9	47.1±0.6	11.5±0.6	32.0±0.2

Table 2.4 Colorimetric parameters (L, a*, b*) for crust and crumb of all the analysed samples*

2.3.5 Compression test results

In addition to the rheological analysis, the best samples from a rheological point of view, were also studied performing lubricated squeeze flow tests. The study of the biaxial flow is important in this kind of systems as it can provide useful information to understand the behaviour of the mixtures during the expansion. Data obtained are reported in terms of biaxial viscosity η_B as a function of strain rate $\dot{\varepsilon}_B$.

The viscosity profiles for all samples were similar. As an example, biaxial extensional viscosity η_B as a function of biaxial strain rate $\dot{\varepsilon}_B$, for BFW-R 1%C, subjected to lubricated squeezing flow at three different crosshead speeds (0.1 mm/s, 0.3 mm/s, 0.5 mm/s), is represented in Figure 2.14
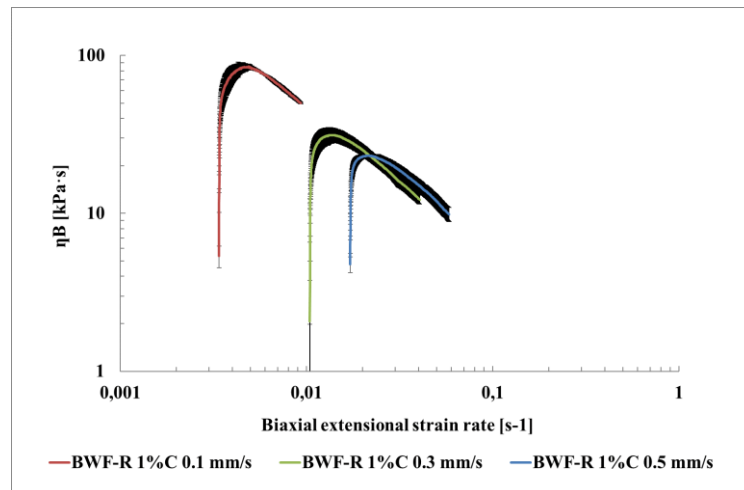
Figure 2.14 η_B as a function of $\dot{\varepsilon}_B$ for the gluten-free dough sample BFW-R 1%C subjected to lubricated squeezing flow at three crosshead speeds.

The relationship between biaxial strain rate and apparent biaxial extensional viscosity, in a double logarithmic plot, for sample BFW-R 1%C, shows a sharp initial rise, followed by a gradual increase in the maximum viscosity and, after that, a slight decrease. All analysed samples showed a pseudoplastic behaviour because viscosity decreased with increasing strain rate.

Comparing in the Figure 2.15 the behaviour of WF and the GF control dough (R,WBF), at the same water content and at the same crosshead speed, is observed that control doughs exhibit a much higher viscosity han the dough prepared with whole wheat flour, as shown in Figure 2.15.
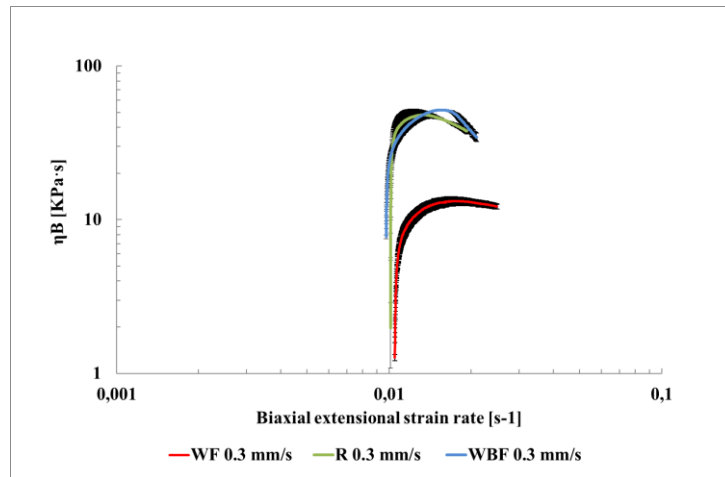
Figure 2.15 η_B as a function of $\dot{\varepsilon}_B$ for samples WF, R and BFW, subjected to lubricated squeezing flow at a compression speed of 0,3 mm/s.

The same behaviour is observed for the samples made with a mixture of rice and buckwheat flour (Figure 2.16) at all the water levels analysed . The viscosity value changed when the water level was increased. The effect of water content is shown in Figure 2.16. Data were compared at the same compression speed of 0,3 mm/s.

From Figure 2.16 it is clear that the gluten-free sample that best approximates its mechanical behaviour to that of the standard is the dough prepared with 85% water and 1% of hydrocolloid, for both CMC (Figure 2.16a) and HPMC (Figure 2.16b). Both types of hydrocolloid, samples WBF-RH1.0 and WBF-RC1.0, containing 75% of water (on flour basis) wich is the same water content of the wheat control dough (WF), exhibited the highest biaxial viscosity. These doughs resulted in a harder and less workable material if compared to the samples prepared using a higher water content.
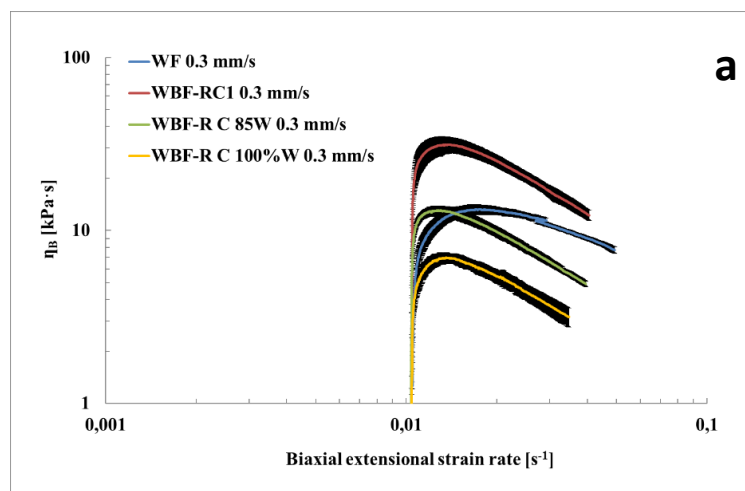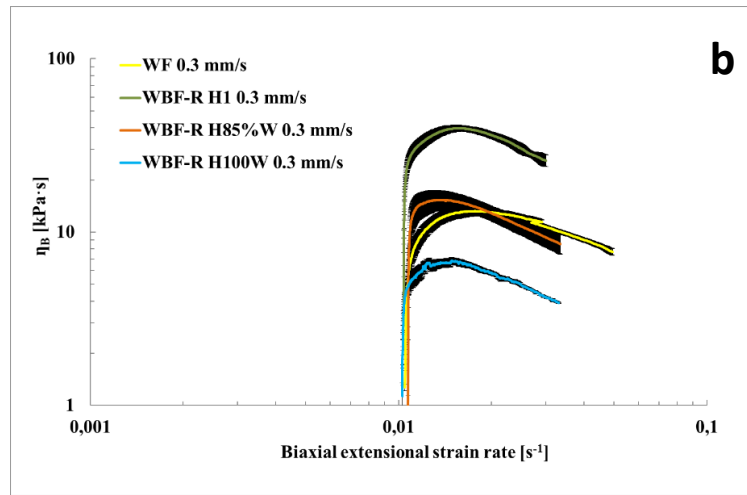
Figure 2.16 η_B *as a function of* ε_B *for samples prepared with CMC (a) and HPMC (b) subjected to lubricated squeezing flow at a compression speed of 0,3 mm/s*

Increasing the amount of water, decreases the overall value of η_B and this behaviour is the same both for samples prepared with HPMC and CMC at all the considered compression speeds. Figure 2.17 shows results for 1%HPMC samples at different water content levels and different crosshead speed.
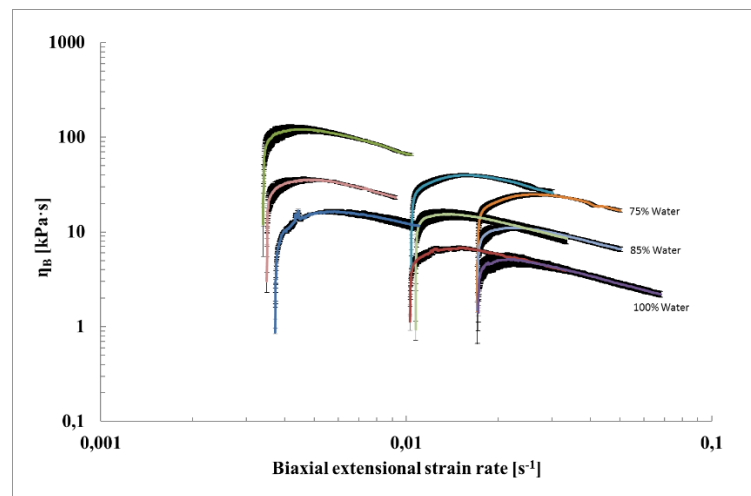
Figure 2.17 η_B *as a function of* $\dot{\varepsilon}_B$ *for the gluten free dough sample prepared with 1% of HPMC and at different water content, subjected to lubricated squeezing flow at three crosshead speeds*

As shown in the previous results, η_B decreases linearly with increasing strain rate ε_B in the double logarithmic plot, appearing as strain rate thinning behaviour which can be fitted by the power law model (Rouille et al., 2005):

$$\eta_B = K\dot{\varepsilon}_B{}^{m-1} (2.7)$$

or

$$\boldsymbol{\sigma_B} = K\dot{\varepsilon}_B{}^m \quad (2.8)$$

where K is the consistency index and *m* is the power-law index. From literature about wheat dough, macroscopic fractures are usually observed around equibiaxial strain (ε_B) values between 0.35–0.40 (Song and Zheng, 2008). To compare the behaviour of the gluten-free doughs in terms of power law parameters, the resultant values of *m* and K are plotted against ε_B.

Power law parameters for the GF control dough (Rm WF, WBF) are shown below in Figure 2.18.
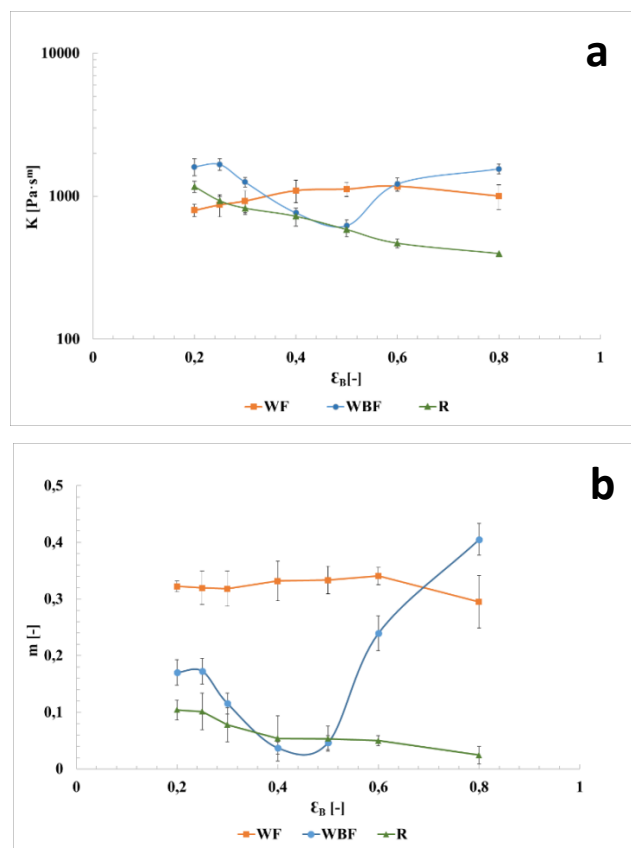
Figure 2.18 a) **K** *parameter as a function of* ε_B *for samples WF, WBF and R; b)* **m** *index as a function of* ε_B *for samples WF, WBF and R*

In a semi-log plot of K vs ε_B (Figure 2.18a), the WF dough consistency (K) slightly increases below $\varepsilon_B \sim 0,3$ while it doesn't change significantly at higher ε_B values. The R dough exhibits an opposite behaviour compared to WF. For the rice dough sample, K decreases gradually, when the biaxial strain increases.

A completely different behaviour is observed for the WBF dough for which K seems to not change for small ε_B, then passes through a minimum with increasing ε_B and then increases again. For $\varepsilon_B > 0,6$, K for WBF does not change significantly.

As far as the power-law index m is concerned (Figure 2.18b), in the wheat flour dough, the average value of m oscillates between 0,3 and 0,4 and seems to be independent of ε_B, thus suggesting that the rate dependence of stress is small. Gluten-free samples show a different behaviour compared to WF.

For the WBF dough, m seems to decrease at small strains, passes through a minimum and then increases again. In the R dough sample, m shows an independency from the equibiaxial strain at small ε_B. A slight decrease of m can be observed at higher ε_B.

From the analysis of the power-law parameters related to the control doughs, prepared at the same water level, it could be concluded that the strain rate dependence is stronger in the WBF dough than in R and WF.

Comparing the power law parameters for the sample prepared using the cellulose derivatives hydrocolloids, for both HPMC (Figure 2.19) and CMC (Figure 2.20), increasing the water content up to 100% (on flour basis) leads to an overall decrease in the consistency index (K) as shown in Figure 2.19a and 2.20a.
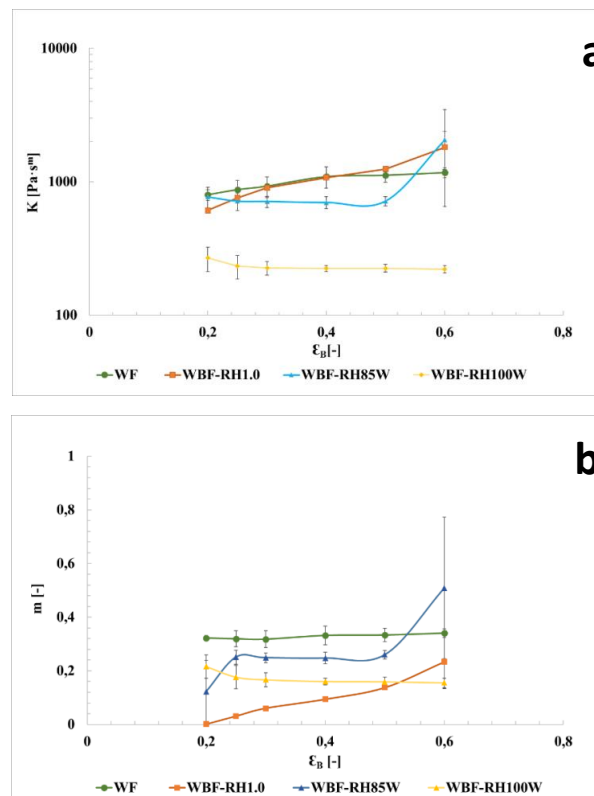
Figure 2.19 a) **K** parameter as a function of ε_B for samples WF, WBF-RH1,WBF-RH85%W, WBF-RH1005W and R; b) **m** index as a function of ε_B for samples WF, WBF-RH1,WBF-RH85%W, WBF-RH1005W

K and m slightly increase when the biaxial strain increases for the WBF-RH1 sample (Figure 2.19a). For WBF-RH85 and WBF-RH100W samples, at $0,2 < \varepsilon_B < 0,5$, K exhibits a linear behaviour with increasing the biaxial strain, while for $\varepsilon_B > 0,5$ an uncertain behaviour can be observed for WBF-RH85 as shown from the standard deviation bar. The WBF-RH85W dough seems to be closer to the WF dough both in terms of K and m.
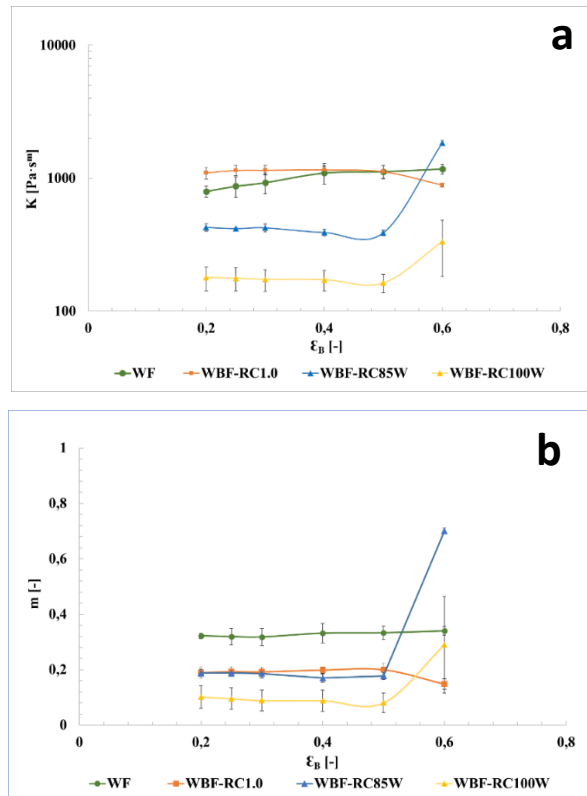
Figure 2.20 *a) **K** parameter as a function of ε_B for samples WF, WBF-RC1,WBF-RC85%W, WBF-RC100%W and R; b) **m** index as a function of ε_B for samples WF, WBF-RC1,WBF-RC85%W, WBF-RC100%W*

Even for the samples prepared with CMC, there isn't a strong ε_B dependency on K and m parameters and also in this case the most similar sample to the WF standard seems to be the WBF-RC85W dough.

2.4. Conclusions

In this work, the effect of cellulose derivatives hydrocolloids and of water content, on buckwheat and rice flour based gluten free bread, were investigated with fundamental rheological analysis tests. Two different hydrocolloids at four concentrations were used in the formulation and for samples considered more interesting two additional water levels were also studied.

Both hydrocolloids significantly modify dough behavior reducing the strength and extension of 3D the network even if more relevant effects are observed mainly for HPMC; the latter, moreover, drastically modifies the dough thermal behavior due to a potential phase separation phenomena that causes a drop in moduli at approximately 61°C.

The increase in the water level leads to a reduction in network strength and extension at room temperature, due to a dilution effect, whereas it yields a relevant increase in network extension at high temperature, probably because the larger amount of available water promotes the structuring phenomena of starch and hydrocolloids.

Obtained results evidenced that the use of proper hydrocolloid concentration with increased water level can lead to GF doughs which are more similar to standard wheat bread. In the present work the best results were obtained with 1% of hydrocolloids and by increasing water to 85% and 100% for CMC and HPMC, respectively.

Positive results were also evidenced by a qualitative visual inspection of breads obtained by doughs at different hydrocolloid and water content. It was observed that the addition of HPMC and CMC improves the internal structure, increasing the porosity even if results comparable to the benchmark were obtained only increasing the water content. Images of internal crumb are in agreement with the results obtained by the rheological analysis, confirming that samples with 85% and 100% of water give very promising results.

Compression tests confirmed that the most prominent effect in the mechanical properties of the dough is due to the water content. Increasing it greatly improves the workability of the gluten free mixture and, as seen from the lubricated squeeze flow measurements, is also confirmed by baking analysis.

It is worth noticing that a finer tuning of both hydrocolloid and water concentration could yield doughs closer to the benchmark and potentially suitable for commercial uses with a good nutritional level due to the high fiber content which meets the current suggestions of the World Health Organization.

References

- Ai, Y. & Jane, J.-l. (2015) Gelatinization and rheological properties of starch. Starch - Stärke, 67(3-4), 213-224.

- Baldino, N., Gabriele, D., Lupi, F. R., de Cindio, B. & Cicerelli, L. (2014) Modeling of baking behavior of semi-sweet short dough biscuits. *Innovative Food Science & Emerging Technologies*, 25, 40-52.

- Barnes, H. A., Hutton, J. F. & Walters, K. (1989) An Introduction to rheology, 1989 edition. The Netherlands: Elsevier Science Publishers B.V., 19-23.

- BeMiller, J. N. (2007). Carbohydrate chemistry for food scientists (No. Ed. 2). American Association of Cereal Chemists, Inc (AACC).

- Bloksma, A. H. (1990). Dough structure, dough rheology and baking quality. *Cereals Food World*, 2, 35, 237-244.

- Champenois, Y., Rao, M. A. & Walker, L. P. (1998) Influence of gluten on the viscoelastic properties of starch pastes and gels. Journal of the Science of Food and Agriculture, 78(1), 119-126

- Costantini, L., Luksic, L., Molinari, R., Kreft, I., Bonafaccia, G., Manzi, L. & Merendino, N. (2014) Development of gluten-free bread using tartary buckwheat and chia flour rich in flavonoids and omega-3 fatty acids as ingredients. *Food Chemistry*, 165, 232-240.

- Fairclough, J. P. A., Yu, H., Kelly, O., Ryan, A. J., Sammler, R. L. & Radler, M. (2012) Interplay between Gelation and Phase Separation in Aqueous Solutions of Methylcellulose and Hydroxypropylmethylcellulose. *Langmuir*, 28(28), 10551-10557.

- Gabriele, D., de Cindio, B., & D'Antona, P. (2001). A weak gel model for foods. *Rheologica Acta*, 40(2), 120-127.

- Gallagher, E., Gormley, T. R., & Arendt, E. K. (2004). Recent advances in the formulation of gluten-free cereal-based products. Trends in *Food Science & Technology*, 15(3), 143-152.

- Guarda, A., Rosell, C. M., Benedito, C., & Galotto, M. J. (2004). Different hydrocolloids as bread improvers and antistaling agents. *Food hydrocolloids*, 18(2), 241-247.

- Hager, A. S., Ryan, L. A., Schwab, C., Gänzle, M. G., O'Doherty, J. V., & Arendt, E. K. (2011). Influence of the soluble fibres inulin and oat β-glucan on

quality of dough and bread. *European Food Research and Technology*, 232(3), 405-413.

- Jekle, M., Mühlberger, K. & Becker, T. (2016) Starch–gluten interactions during gelatinization and its functionality in dough like model systems. *Food Hydrocolloids*, 54, Part A, 196-201.

- Kokelaar, J. J., Van Vliet, T., & Prins, A. (1996). Strain hardening properties and extensibility of flour and gluten doughs in relation to breadmaking performance. *Journal of Cereal Science*, 24(3), 199-214.

- Lazaridou, A., Duta, D., Papageorgiou, M., Belc, N., & Biliaderis, C. G. (2007). Effects of hydrocolloids on dough rheology and bread quality parameters in gluten-free formulations. *Journal of food engineering*, 79(3), 1033-1047.

- Lupi, F. R., Gabriele, D., De Cindio, B., Sánchez, M. C., & Gallegos, C. (2011). A rheological analysis of structured water-in-olive oil emulsions. *Journal of Food Engineering*, 107(3), 296-303.

- Marco, C., & Rosell, C. M. (2008). Breadmaking performance of protein enriched, gluten-free breads. *European Food Research and Technology*, 227(4), 1205-1213.

- Marino, R., Giovando, S. & Gabriele, D. (2014) Effect of tannin addition on the rheological properties of starch-based adhesives. *Applied Rheology*, 24, 46138.

- Mariotti, M., Pagani, M. A., & Lucisano, M. (2013). The role of buckwheat and HPMC on the breadmaking properties of some commercial gluten-free bread mixtures. Food Hydrocolloids, 30(1), 393-400.

- Migliori, M. & Gabriele, D. (2010) Effect of pentosan addition on dough rheological properties. *Food Research International*, 43(9), 2315-2320.

- Migliori, M., Gabriele, D., Baldino, N., Lupi, F. R. & de Cindio, B. (2011) Rheological Properties of Batter Dough: Effect of Egg Level. *Journal of Food Process Engineering*, 34(4), 1266-1281.

- Miyazaki, M., & Morita, N. (2005). Effect of heat-moisture treated maize starch on the properties of dough and bread. *Food Research International*, 38(4), 369-376.

- Mohammadi, M., Sadeghnia, N., Azizi, M. H., Neyestani, T. R., & Mortazavian, A. M. (2014). Development of gluten-free flat bread using hydrocolloids: Xanthan and CMC. *Journal of Industrial and Engineering Chemistry*, 20(4), 1812-1818.

- Ng, T. S. K. & McKinley, G. H. (2008) Power law gels at finite strains: The nonlinear rheology of gluten gels. *Journal of Rheology*, 52(2), 417-449.

- Onyango, C., Unbehend, G., & Lindhauer, M. G. (2009). Effect of cellulose-derivatives and emulsifiers on creep-recovery and crumb properties of gluten-free bread prepared from sorghum and gelatinised cassava starch. *Food Research International*, 42(8), 949-955.

- Peressini, D., Sensidoni, A., Pollini, C. M., Gabriele, D., Migliori, M. & de Cindio, B. (2002) Filled-snacks production by co-extrusion-cooking. Part 3. A rheological-based method to compare filler processing properties. Journal of Food Engineering, 54(3), 227-240.

- Phimolsiripol, Y., Mukprasirt, A., & Schoenlechner, R. (2012). Quality improvement of rice-based gluten-free bread using different dietary fibre fractions of rice bran. *Journal of Cereal Science*, 56(2), 389-395.

- Rao, M. A. (1999) Rheology of fluid and semisolid foods Principles and Applications. Gaithersburg: Aspen Publishers, Inc.

- Rouillé, J., Della Valle, G., Lefebvre, J., & Sliwinski, E. (2005). Shear and extensional properties of bread doughs affected by their minor components. *Journal of Cereal Science*, 42(1), 45-57.

- Rojas, J. A., Rosell, C. M., & De Barber, C. B. (1999). Pasting properties of different wheat flour-hydrocolloid systems. *Food hydrocolloids*, *13*(1), 27-33.

- Sabanis, D., & Tzia, C. (2011). Selected structural characteristics of HPMC-containing gluten free bread: a response surface methodology study for optimizing quality. *International journal of food properties*, 14(2), 417-431.

- Sabanis, D., Lebesi, D., & Tzia, C. (2009). Effect of dietary fibre enrichment on selected properties of gluten-free bread. LWT-Food Science and Technology, 42(8), 1380-1389.

- Sciarini, L. S., Ribotta, P. D., León, A. E., & Pérez, G. T. (2010). Influence of gluten-free flours and their mixtures on batter properties and bread quality. Food and Bioprocess Technology, 3(4), 577-585.

- Sciarini, L. S., Ribotta, P. D., León, A. E., & Pérez, G. T. (2012). Incorporation of several additives into gluten free breads: Effect on dough properties and bread quality. *Journal of Food Engineering*, 111(4), 590-597.

- Singh, N., Singh, J., Kaur, L., Sodhi, N. S. & Gill, B. S. (2003) Morphological, thermal and rheological properties of starches from different botanical sources. Food Chemistry, 81(2), 219-231.

- Skrabanja, V., Liljeberg Elmståhl, H. G., Kreft, I., & Björck, I. M. (2001). Nutritional properties of starch in buckwheat products: studies in vitro and in vivo. *Journal of Agricultural and Food Chemistry*, 49(1), 490-496.

- Song, Y., & Zheng, Q. (2008). Influence of gliadin removal on strain hardening of hydrated wheat gluten during equibiaxial extensional deformation. *Journal of cereal science*, 48(1), 58-67.

- Soskey, P. R., & Winter, H. H. (1985). Equibiaxial extension of two polymer melts: polystyrene and low density polyethylene. *Journal of Rheology*, 29(5), 493-517.

- Takahashi, M., Isaki, T., Takigawa, T., & Masuda, T. (1993). Measurement of biaxial and uniaxial extensional flow behavior of polymer melts at constant strain rates. *Journal of Rheology*, 37(5), 827-846.

- Thompson, T., Dennis, M., Higgins, L. A., Lee, A. R., & Sharrett, M. K. (2005). Gluten-free diet survey: are Americans with coeliac disease consuming recommended amounts of fibre, iron, calcium and grain foods? *Journal of Human Nutrition and Dietetics*, 18(3), 163-169.

- Tian, Q., Li, D., & Patil, B. S. (2002). Identification and determination of flavonoids in buckwheat (Fagopyrum esculentum Moench, Polygonaceae) by high-performance liquid chromatography with electrospray ionisation mass spectrometry and photodiode array ultraviolet detection. *Phytochemical Analysis*, 13(5), 251-256.

- Watanabe, M., Ohshita, Y., & Tsushida, T. (1997). Antioxidant compounds from buckwheat (Fagopyrum esculentum Möench) hulls. *Journal of Agricultural and Food Chemistry*, 45(4), 1039-1044.

- Wu, K., Gan, R., Dai, S., Cai, Y.-Z., Corke, H. & Zhu, F. (2016) Buckwheat and Millet Affect Thermal, Rheological, and Gelling Properties of Wheat Flour. Journal of Food Science, 81(3), E627-E636.

Study of Gluten-free bread formulations based on rice and buckwheat flours: Part II

Abstract

In this second part, GF bread doughs based on buckwheat and rice flour, were further analysed using additional evaluative techniques, allowing integration of the information with that gained from the empirical and fundamental rheological measurements discussed in Chapter 2.

The aim of the study was to deepen the knowledge of the effect of cellulose derivatives hydrocolloids (CMC and HPMC) and of the water content, on the final structure of the product to determine a relationship with the physico-chemical properties of the material, which were explored by investigating water partitioning in the bread dough during heating and interaction of the baking ingredients.

Bread dough is formed by several aqueous phases, each rich in a given component of the dough, (starch granules, globular proteins, non-starch carbohydrates, etc.). This is further supported by the description of bread dough as a metastable dispersed system with a huge interphase surface across which water can move from one phase to another (Tolstoguzov, 1997). In this study, the water distribution in the bread dough was characterized using thermogravimetric analysis. Changes taking place in the dough, after heating and the influence of structure improver ingredients, were monitored performing IR spectroscopic analysis.

Additional investigations into the microstructure of the final baked doughs were also performed. Porosimetry on dried crumb were conducted on all samples, trying to adapt the low-temperature nitrogen adsorption techniques, to the analysis of the microstructure of these materials, with the aim of elucidating surface characteristics, which is important for predicting the interactions between water and food components. Moisture adsorption and/or desorption occurs in fact on the surface of food, impacting its shelf life. GF products are known to be prone to a shorter shelf-life if compared to the traditional wheat equivalent and therefore need to be

preserved in special packaging conditions which negatively impacts the final cost of the product.

On same samples, SEM analyses were carried out to further explore the characteristic surface morphology of these systems.

3.1 Introduction

Different investigation techniques offer their own insight into a material's properties, enhancing the detection of some phenomena while they may be unable to shed light on others, therefore, for a comprehensive understanding of a material's character, usually more than just one analytical technique is required. Their combined use can provide synergistic advantages for monitoring and characterizing changes in physical or chemical structure. Product quality, and performance during processing, or in the final application, not only depends on the material structure, but also on temperature/time dependent changes and external conditions (inert or oxidizing atmosphere, pressure, relative humidity).

Techniques of major interest to food researchers, which complement fundamental rheological measurements, include empirical thermal analyses (thermogravimetric, differential scanning calorimetry), FT-IR spectroscopy, and microscopy. By understanding structure, and related physio - chemical properties in foods, formulations can be developed to provide desired end-use properties (e.g. softness, crispness, fast dissolution rate, extended shelf life etc.) (Nielsen, 2010). Scientific results related to the application of rheology, thermal analysis and FTIR used to investigate GF bread model systems were reported in Chapter 1, (Section 1.6, 1.7, 1.8).

Despite several previous works, the study of food systems, using available instrumentation and investigation techniques, has encountered many difficulties both in the choice of set-up conditions and in the interpretation of data. The advantages of adapting modern instruments to the study of food systems, and the associated disadvantages that make the research in this field complex, are highlighted below. Taking them into account will help in reading the results in the following sections.

Thermogravimetric analysis (TGA) is a widely used thermal analysis method, used to characterize food materials. Through TGA, it is possible to detect and quantify the presence of bulk water and/or associated water (Fessas and Schiraldi, 2001), and to identify the temperature at which molecular decomposition (chemical changes) begin (Liu et al., 2010), but most TGA instruments, have several natural limitations (Nielsen, 2010). Although TGA provides a quantitative measurement of weight change, it is often difficult to quantify the weight loss related to a specific component or water fraction, because weight losses typically overlap in

temperature (Nielsen, 2010). Slowing the heating rate and reducing the sample weight could be ways to improve thermal resolution of these weight losses. However, lowering the heating rate, extends the time of measurement, while using less sample reduces the accuracy of small weight changes.

Another limitation of TGA is that it cannot identify the chemistry of gases evolved from the sample. Knowledge of the gas composition could help to distinguish between water loss and loss of low molecular weight additives, such as flavours and fragrances, and help to determine the mechanism involved in cooking and decomposition. Off-gases could be chemically identified using interface devices (such as gas chromatography, FTIR etc.) between the gas product and the TGA.

Rheology allows the study of steady-state and time-dependent viscoelastic properties of a material, as a function of stress and/or strain. However, the viscoelastic properties of a material mutually depend on the structure and, especially, the structural changes of the material at the molecular level. IR spectroscopy is, therefore, an excellent tool for determining the identity and quantity of molecules in a sample, allowing comparison of chemical information with rheological properties. Mid-infrared (M-IR) spectroscopy is widely used in the chemical industry and analytical laboratory. The M-IR region rages from 4000 to 400 cm^{-1}, which is useful for the study of organic compounds (Guillen and Cabo, 1997). However, the application of this technique to food samples has only been seriously considered recently due to instrument improvements (i.e. the development of Fourier-transform instruments), as well as the use of powerful mathematical techniques allowing the extraction of valuable information from complex data, and new sampling options.

The limiting factor in using M-IR spectroscopy with food systems is due to the fact that foods are highly scattering and contain water, which is a very strong infrared absorber. Consequently, water is a significant absorber in the M-IR spectral region and can interfere with the determination of other components present in food systems (Karoui et al., 2010). The M-IR region has been applied to study the secondary structure of food proteins (Carbonaro and Nucara, 2010) or to explore structure-quality relationships in food systems (Karoui et al., 2010), however, foods still represent a significant analytical challenge. They are highly complex, variable and can be found in many different physical states. Water, carbohydrates, proteins, and fats are the main components of food systems; other constituents, such as vitamins, minerals, etc., are present at lower concentrations. These minor components, may contribute to the shape of the absorbance spectrum obtained in the M-IR region, although, in practice, the major components (water, carbohydrates, proteins, fats) dominate, because constituents present

at concentrations below ~0.1% w/w are difficult to detect in water-rich systems (Karoui et al., 2010).

Food heterogeneity results in considerable spectral complexity and conventional approaches to the use of spectra for, e.g. quantitative analysis, may not be applied; also, specificity in band assignment may be lost when a sample contains many chemically similar components (e.g. sugars). Taking into account all the limitations arising from the use of the evaluative techniques discussed above and applied to food systems, the interpretation of the results from TGA, FT-IR spectroscopy an BET analysis will be shown in this chapter.

3.2 Materials and Methods

3.2.1 Sample Preparation

Thermal Analysis

Due to the small amount of sample required for thermogravimetric analysis, lab scale GF-bread doughs, using the same ingredients and proportions reported in Chapter 2 (Section2.2, Table 2.2), were obtained using a simple standardized procedure, by manually mixing all ingredients for 10 min in a beaker ensuring homogeneity of the sample upon completion of the mixing stage. About 30 mg of the fresh dough was used for TGA analysis.

FT-IR Analysis

Dried samples, obtained from TGA measurements, were subjected to FT-IR analysis; sample mass was kept constant (~ 15 mg) for these studies.

BET Analysis

Well-dried bread crumb was subjected to BET surface area analysis. Lab-scale bread samples were prepared using the fresh dough obtained for thermal analysis. After mixing, the dough was left at 40 ˚C for 1 hr to prove before baking, in a preheated oven, at 185 ˚C for 45 min.

After baking and subsequent cooling, samples of fresh crumb were pre-dried in a vacuum oven at 40 °C for 24 hrs as a first sample drying, and speeding up the degas stage that precedes porosimetry measurement.
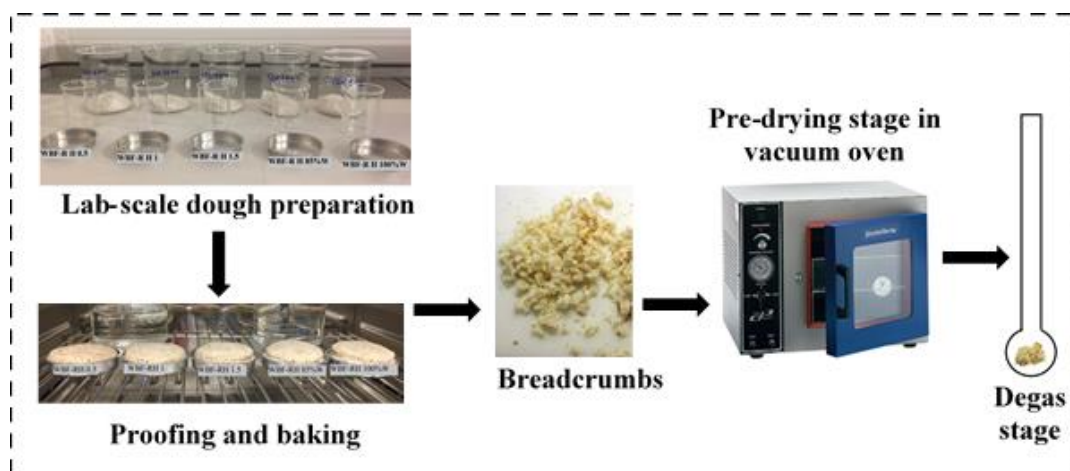
Figure 3.1 - preparation of bread crumb for BET analysis

3.2.2 Experimental runs

3.2.2.1 Thermal Analysis

Thermal analysis was carried out using a Simultaneous Thermal Analyser (STA 449 F1 Jupiter®, NETZSCH, Germany) and 20 to 30 mg of dough sample, placed in an unsealed aluminium pan. A second, empty aluminium pan was used as a reference.
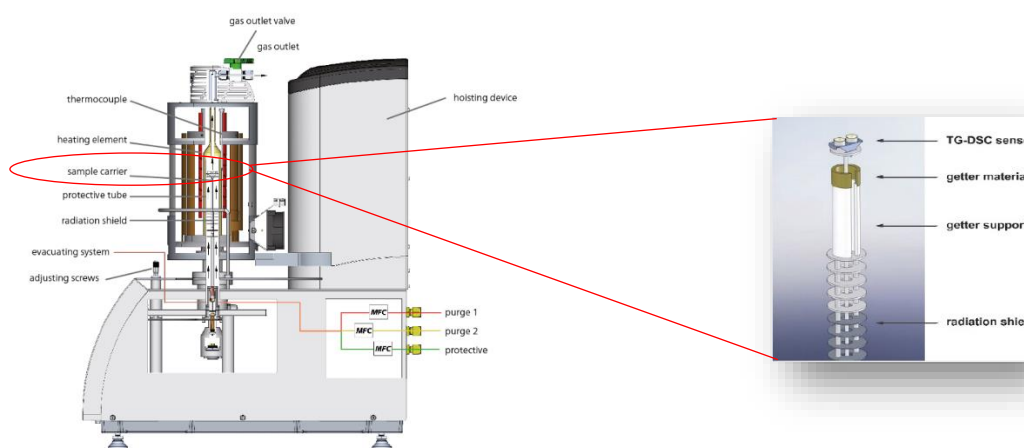
Figure 3.2- STA (Simultaneous Thermal Analyser) 449 F1 Jupiter®

Both the sample and empty reference pans were placed in the furnace unit. Runs from 298 K to 473 K were carried out at a heating rate of 5 K/min in an oxidative atmosphere. (Fessas and Schiraldi 2001, Fessas and Schiraldi 2005, Lodi and Vodovotz 2008). All samples were analysed in triplicate.

The DTG traces, derived from the TGA data, were subjected to baseline correction and deconvoluted by a non-linear regression curve fitting program of Gaussian peaks using the PeakFit software (Origin® Pro 2015 Graphs & Analysis). The deconvoluted DTG curve was used to differentiate between the kinematic energy states of water in the dough.

3.2.2.2 Spectroscopic Analysis

The dried samples used for thermal analysis were also subjected to FTIR analysis. FTIR spectra were obtained using an ABB Bomem MB 3000 Fourier Transform Near Infrared Spectrometer, with spectra collected over the range 4000-400 cm^{-1} at a resolution of 4 cm^{-1}.

Due to the fact that, for larger molecules, significant information can be hidden under broad and featureless absorption bands (Barth 2007), a curve fitting procedure was used to deconvolute the amide I and amide II bands in the region 1500-1800 cm^{-1}, with the aim of resolving any hidden peaks and gaining information about the changes in the protein secondary structural component of the samples, as well as about possible interactions of the protein-polysaccharides.

Raw absorbance spectra were smoothed using a nine-point Savitsky-Golay smoothing function. Normalised spectra were subjected to a multi-point elastic base-line correction (Sivam et al. 2013), and the treated spectra were deconvoluted by a non-linear regression curve fitting program of Gaussian peaks. All treatments were carried out using Origin® Pro 2015 Graphs & Analysis. Best curve-fitting was obtained at the lowest possible χ^2 value.

3.2.2.3 Porosimetry

The gas adsorption techniques used here are based on the interpretation of adsorption and desorption isotherms for probe gases on the surface of solids, and they are mostly used for the characterisation of meso and microporous materials. The basic idea is that when gas molecules contact a solid surface, the gas will adsorb onto the surface in quantities that are a function of its partial pressure in the bulk. The measurement of the amount of gas adsorbed over a range of partial pressures at a single temperature results in a graph known as an adsorption isotherm.

The interpretation of isotherms happens according to different methods, but the most commonly used is the so-called BET theory, which was originally proposed by (Brunauer, Emmett and Teller 1938). The method determines the specific surface area of solid materials based on the principle of the number of gas molecules that are necessary and sufficient to cover all of the available surface of the material in one layer (BET surface area). The technique also allows determination of the total pore volume, median pore size and pore volume distribution,

as well as predominant pore shapes, and the inception point and magnitude of hysteresis, in the case of mesoporous materials.

During adsorption, gases are dosed onto the adsorbent surface in increasing pressure steps. At each step, the system is allowed to equilibrate, whereupon the maximum amount of gas has been adsorbed at the applied pressure. Dividing each absolute pressure step value by the saturation pressure, results in a relative pressure scale for the system, with values ranging between 0 and 1. At constant temperature, the relationship between the amount of gas adsorbed by a surface and the relative pressure of the gas is defined, by I.U.P.A.C., as an adsorption isotherm. As different gases and surfaces interact in a variety of ways, the shape of the adsorption isotherm can vary correspondingly. For this reason, I.U.P.A.C. has classified six isotherm Types, the shape of which are dependent on the interactions occurring (Thommes et al. 2015). These are shown in Figure 3.3.
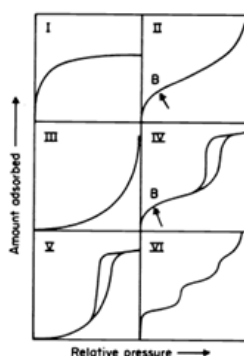
Figure 3.3 - Adsorption isotherms as classified by IUPAC

Many parameters that describe the pore structure of a sample, including specific surface area, pore volume, and pore size distribution, can be determined using BET Analysis. For the samples studied here, the most significant parameter will be the specific surface area.

Degas Settings

A degassing step was performed in the degas station of the *Micrometrics ASAP 2420 apparatus* (Figure 3.4) in order to clean the surface prior to adsorption analysis. Samples were degassed by heating to 40 °C while reducing the pressure, first to 5 mmHg, followed by a further reduction to 10 µmHg. Upon reaching this second pressure the sample temperature was held at 40°C for another 1hr. Upon completion of the degas cycle, samples were cooled to room temperature and backfilled with pure nitrogen.
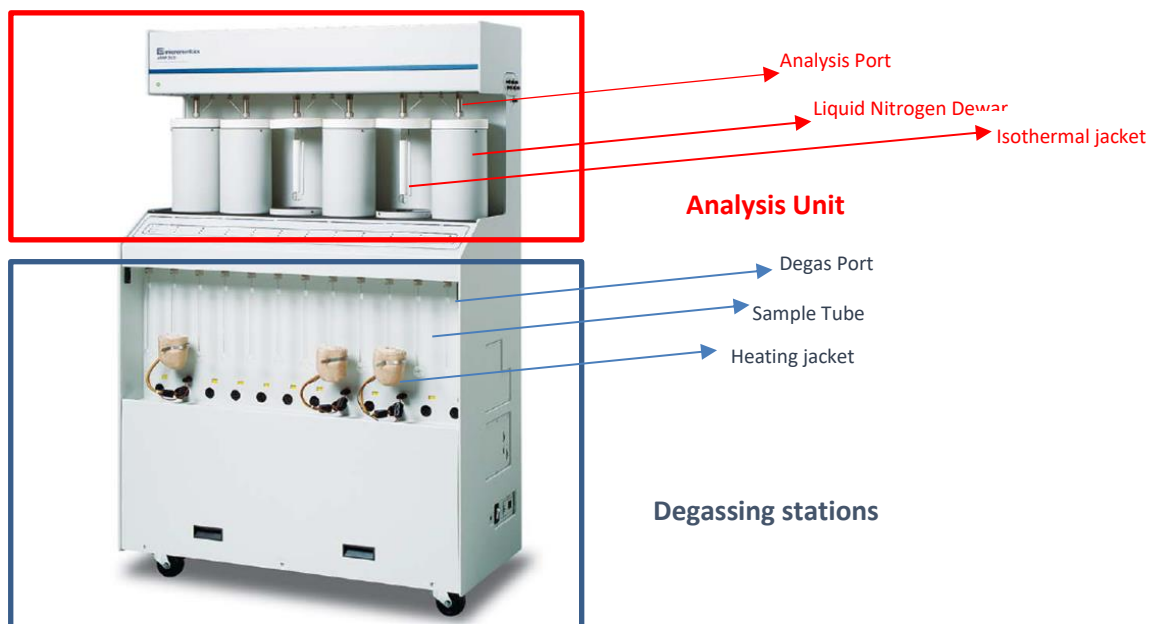
Figure 3.4 Micrometrics ASAP 2420 apparatus

Experimentally, approximately 5 g of the sample to be analysed was weighed into a sample tube (Figure 3.5) and the tube was attached to a degas port on the ASAP instrument. The bulb of the tube was inserted into a heating jacket, ensuring contact between the internal thermocouple and the glass.

Figure 3.5 - Sample tube

After degassing, samples were reweighed to account for any mass loss that occurred during degassing. The new mass was subsequently used in the settings for BET-analysis.

BET-Analysis

The analysis cycle consisted of 40 nitrogen adsorption steps in the relative pressure range of 0.01 - 1, followed by a 30-point desorption branch, conducted between the relative pressures of 1 - 0.1.

Determination of BET Specific Surface Area

Two of the most commonly used models, which have been developed to explain the adsorption of molecules onto a solid surface are the Langmuir and Brunauer – Emmett -Teller (BET) models.

Confining adsorption to a monolayer, the Langmuir equation (Langmuir, 1916) can be written as:

$$\frac{V}{V_m} = \frac{Kp}{1+Kp} \; (Eq.3.1)$$

Where:

- p is the adsorbate equilibrium pressure;
- V is the volume of gas adsorbed;
- V_m is the volume of gas required to complete monolayer coverage;
- K is a constant for any given gas-solid pair.

Rearranging the equation 3.1 in a form of a straight line:

$$\frac{p}{V} = \frac{1}{KV_m} + \frac{p}{V_m} \; (Eq.3.2)$$

A plot of p/V vs. p will therefore have a gradient of 1/Vm, from which the monolayer volume can be calculated.

The specific surface area (Sa [$m^2 \cdot g^{-1}$]) of the material can be calculated from the with the following equation:

$$S_a = n_m L A_m \; (Eq.3.3a)$$

where:

- A_m is the cross-sectional area of a gas molecule, i.e. the area on the surface it occupies ($1.62 \cdot 10^{-19}$ m^2 molecule^{-1} for the most commonly used gas, nitrogen);
- L is the Avogadro's constant ($6.022 \cdot 10^{23}$ molecule mol^{-1});
- n_m is the monolayer capacity [$mol_{sorbate}/g_{solid}$] defined as the amount of adsorbate that can be accommodated in a completely filled monolayer on the surface of one gram of solid (Averill et al., 1999). It can be computed from Vm as:

$$n_m = \frac{V_m}{V} \; (Eq. \; 3.3b)$$

Where Vm ($m^3 \cdot g^{-1}$) is the volume of the adsorbate monolayer per gram of adsorbent and V is the gas molar volume at standard temperature and pressure (i.e. 0,022414 m^3/mol)

Brunauer, Emmett and Teller expanded on the Langmuir equation, with a theory that could also account for multilayer adsorption. Some additional assumptions were required when first developing the theory:

- the uppermost layer of adsorbed molecules is in equilibrium with the bulk gas;
- the first adsorbed layer has a heat of adsorption, while subsequent layers have a heat of condensation;
- at saturation pressure, an infinite number of layers become adsorbed onto the surface.

The BET equation is most commonly used in the linearized form:

$$\frac{p}{V(p_0-p)} = \frac{1}{V_m C} + \frac{(C-1)p}{p_0 V_m C} \quad (Eq.3.4)$$

Where:

- p is the equilibrium pressure;
- p_0 is the saturation pressure;
- V is the volume of gas adsorbed at standard temperature and pressure (STP);
- Vm is the volume adsorbed to complete the monolayer;
- C is a constant related to the adsorbate-adsorbent interaction energy.

A plot of $\frac{p}{V(p_0-p)}$ vs $\frac{p}{p_0}$ (Figure 3.6) yields a plot with slope $\frac{(C-1)}{V_m C}$ and intercept $\frac{1}{V_m C}$, from which values of V_m and C may be calculated.
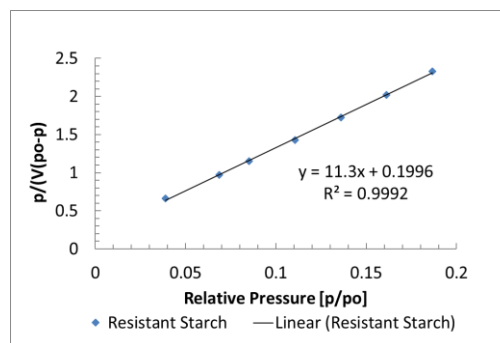
Figure 3.6 Plot of $\frac{p}{V(p_0-p)}$ vs $\frac{p}{p_0}$

Calculation of these values requires a linear relationship between the variables. To ensure a linear section of the graph is analysed, the relative pressure (p/p_0) range over which the data

can be plotted is limited to between 0.05 and 0.3. This linear portion is known as a BET plot (Sing, 1985). After determining the value of V_m the specific surface area of the sample can be estimated again.

3.2.2.4 SEM analysis of bread structure

The surface morphology of the small portions of bread, previously pre-dried for porosimetry measurements, were analysed using a Field Emission Scanning Electron Microscope (FE-SEM) HITACHI SU-6600). Due to the non-conductive nature of the materials, prior to analysis, samples were coated with a conductive layer of gold to enable and/or improve SEM imaging.

3.3 Results and discussion

3.3.1 Thermogravimetric Analysis (DTG)

The chemical, physical and viscoelastic properties, of the final starchy products, depend mainly on the extension of water-biopolymer interactions developed during processing and baking (Moreira, Chenlo and Arufe, 2015). When heating carbohydrate systems, weight loss is mainly attributed to moisture loss (Fessas and Schiraldi 2001). In a dehydration process, the TG trace describes how much water is lost and how quickly it is released as the system is heated at a constant heating rate.

In the case of water vaporization from food samples, it is easy to verify that the corresponding enthalpy is close to 2.3 kJ/g (with reference to the mass of water released), which is the vaporization enthalpy of pure water in the considered temperature range. This can be simply evaluated from the simultaneous DSC trace recorded during measurements. Data obtained from a DSC trace allows the user to establish which peaks resulting from the studied materials are related to water vaporization, being those with enthalpies of approximately 2.45 kJ/g, as evaluated from the example of the WBF-R sample.

In this section, only results obtained from thermogravimetric analysis will be discussed. DSC curve analysis is omitted, as the main goal of the work is to study how the composition of the system affects the water distribution in the samples by analysing water loss with increasing temperature, and neglecting the heat associated with vaporization of the different water fractions, which is difficult to detect in these systems where different transitions occur and overlap in the same temperature range.

Since consumer expectations for gluten-free bread are greatly influenced by traditional bread attributes, the samples were analysed, and the results compared to a wheat flour bread control sample (WF).

3.3.1.1 Effects of different flours

All analysed GF samples showed similar TGA traces and those for samples WF, WBF and R, which have the same water content, are compared in Figure 3.7.
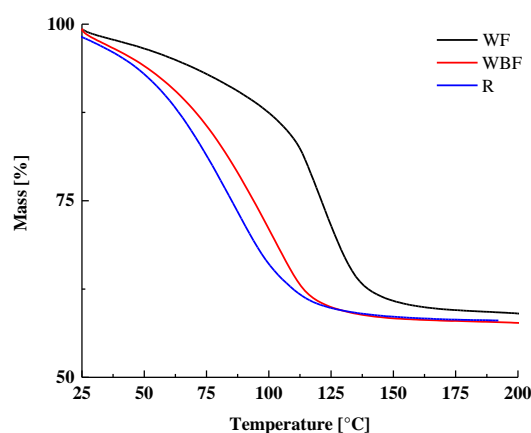
Figure 3.7 - TGA curves for samples WF, WBF and R

When the mass loss is mainly due to a single process, such as dehydration in carbohydrate systems (Fessas and Schiraldi 2001), the TG trace shows a sigmoidal descending trend with a flexus at some intermediate temperature where the water loss rate is at a maximum, as shown in Figure 3.7.

It can be seen that the significant weight loss, as detected by TGA, occurred in a different temperature range for the two types of doughs (glutenous and gluten-free). For the glutenous sample (WF), the maximum weight loss occurred in the temperature range 110-140˚C, which is a relatively higher temperature when compared to the GF samples, for which the maximum weight loss occurs between 75-100˚C. In addition, the maximum weight loss for the WBF sample seems to be shifted to a slightly higher temperature than the R sample, prepared with rice flour only.

Overall, water seems to be released at lower temperatures, and at a faster rate, in the GF doughs than for WF, confirming that the presence of the gluten network may present a barrier to water loss (Crockett et al., 2001), preventing the bread from dehydrating quickly. On the

other hand, the differences between the WBF and R samples could be explained by considering the composition of both GF flours. Nutritional information for both flours is reported in Table 3.1. Buckwheat flour is high in fibre and proteins, with a flour protein content closer to wheat flour. Both protein and fibre have water holding abilities, which could explain the different behaviour observed when compared to the R sample but, although buckwheat is considered a pseudo-cereal, the high proportion of albumins plus globulins (70%) and the low proportion of ethanol and alkali soluble proteins (21%), which are similar to the wheat proteins, indicate that in the buckwheat flour, the proteins are more similar to those of other dicotyledonous seeds (Zheng et al., 1997). Indeed, buckwheat protein has water holding and emulsifying capacities closer to soy proteins (Zheng et al., 1997).

Typical Values	Wholemeal Plain Flour (Allinson)	Buckwheat Flour (Nutrifree)	Rice Flour (Nutrifree)
	Typical Values Per 100g	Typical Values Per 100g	Typical Values Per 100g
Energy	350 kcal	359 kcal	353 kcal
Fat	2.6 g	2.1 g	1.1 g
Of which saturates	0.5 g	0.4 g	0.3 g
Carbohydrate	65 g	70.1 g	78.2 g
Of which sugars	0.8 g	0.6 g	0.3 g
Fibre	10 g	7.5 g	1.6 g
Protein	12 g	11.2 g	7.1 g
Salt	<0.03 g	0.004 g	0.29 g

Table 3.1 Nutritional fact of wheat flour, buckwheat flour and rice flour

Protein content impacts on the water distribution in the sample. Proteins can indeed build a "barrier" of sorts, surrounding starch, and limiting water diffusion in the dough, and to the starch granules, therefore, slowing gelatinization. This effect is related not only to gluten but is also observed in other starch-protein systems (Jekle et al, 2016).

Comparing the temperature range of the maximum weight loss for the samples studied, TG results obtained for the control breads (WF, R, WBF), confirmed the trend of the onset gelatinization temperature (T_o) (i.e. WF>WBF>R) obtained from rheological analysis. Sample R is indeed characterized by the lowest temperature range but also the lowest T_o value, (Section

2.3.2) probably a result of the low fibre and protein contents that do not hinder gelatinization, as discussed in Chapter 2, allowing water to evaporate easily.

3.3.1.2 Effect of Hydrocolloids concentration

From rheological analysis, sample WBF-R, (70% Rice flour, 30% Buckwheat flour) was chosen as a promising starting point for hydrocolloid addition. It exhibits rheological properties that are not so different to WF, both in terms of complex modulus and phase angle at different temperatures. Therefore, hydrocolloid addition could be helpful in reducing the differences with respect to WF, finally yielding a potential GF bread with high fibre content as discussed in Chapter 2.

3.3.1.3 Effects of HPMC addition

TG Results obtained for samples produced using different HPMC concentration are shown in Figure 3.8. Adding the HPMC hydrocolloid had an impact on the maximum weight loss temperature range and, therefore, the water mobility in the material.
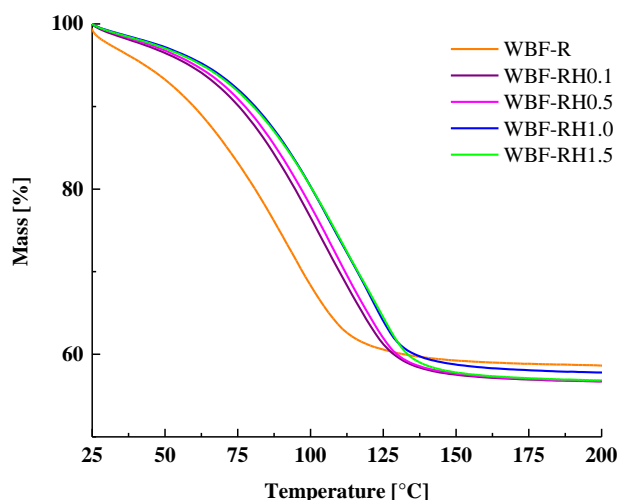
Figure 3.8 - TGA curves for samples WBF, WBF-RC 0.1, WBF-RC 0.5, WBF-RH 1, WBF-RC 1.5

The results obtained for the WBF-R sample show that water is released at lower temperatures compared to the samples containing HPMC at different levels. This result could confirm the water retention ability of the added hydrocolloid.

Increasing the amount of the HPMC up to 1% (w/w% on flour basis), seems to slightly improve water retention in the samples at higher temperature. No differences can be observed between the samples at 1% and 1.5%. Also, in this case, a correspondence with the rheological results can be observed. When the onset gelatinization temperature (T_o) is considered, it can be seen that structuring phenomena are shifted towards higher temperature, when adding HPMC, probably due to competition for water availability between starch and HPMC, as discussed in Chapter 2.

3.3.1.4 Effects of CMC addition

A similar behaviour is observed for the CMC hydrocolloid. Also in the case of CMC, samples showed the significant weight loss at higher temperatures compared to the control bread.
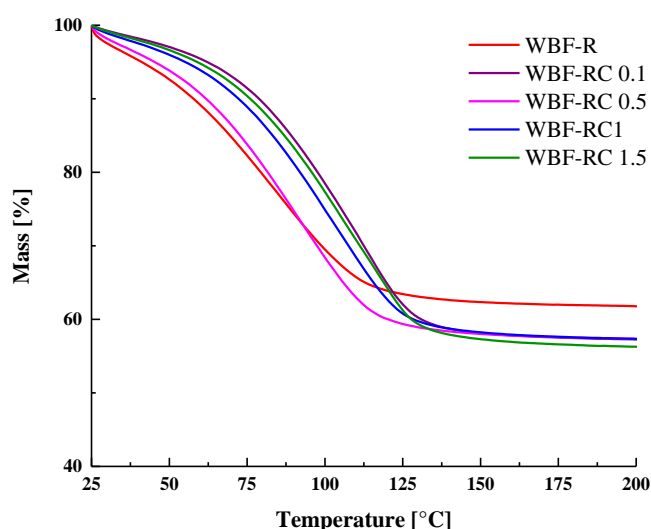
Figure 3.9 - TGA curves for samples WBF, WBF-RC 0.1, WBF-RC 0.5, WBF-RH 1, WBF-RC 1.5

While a similar temperature range can be observed for the maximum weight loss-region for samples at 0.1%, 1% and 1.5% CMC, a slightly shifted range to lower temperatures can be observed for the sample at 0.5%.

3.3.1.5 Effect of water content

From a rheological point of view, the most promising doughs, as potential bases for gluten-free bread, are the ones prepared with higher hydrocolloid addition, i.e. 1% and 1.5%, as discussed in Chapter 2. Differences in thermal behaviour, as also confirmed by

thermogravimetric analysis, are quite small and, with the aim of reducing the amount of additives (also for economic reasons), samples with 1% of additives were chosen as a starting point to investigate the effects of different water levels, increasing from 70% (used for all samples) to 85% and then to 100%. All percentages refer to the total amount of flour, as reported in Table 2.2. Changing the amount of water affected the slope of the TG trace, as shown in Figure 3.10.
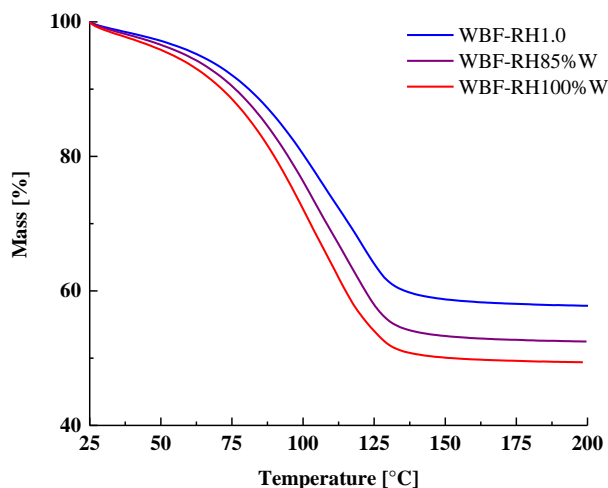
Figure 3.10 - TGA curves for samples WBF-RH 1, WBF-RH 85%W, WBF-RH 100%W

The increased level of available water, in the sample containing HPMC, causes water loss at lower temperatures, although the trend found for HPMC samples is not the same as for CMC, as shown in Figure 3.11. While adding more water shifted the maximum weight loss of WBF-RC85%W to lower temperatures, and increased the slope of the TG trace relative to WBF-RC 100%W, as also shown in Figure 3.11.
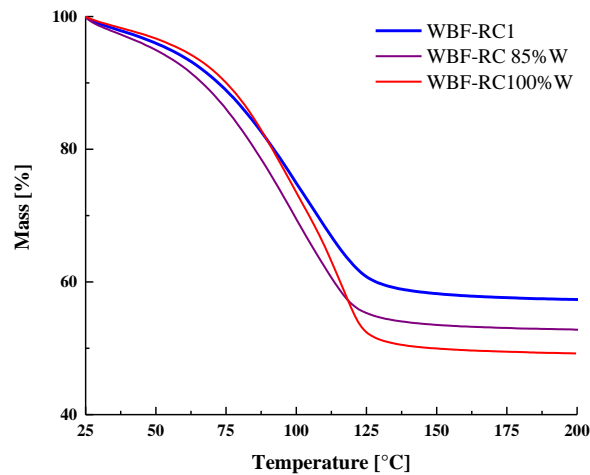
Figure 3.11 - TGA curves for samples WBF-RC 1, WBF-RC 85%W, WBF-RC 100%W

The properties of hydrocolloids vary to a great extent, depending on the origin and chemical structure of the material (Rojas, Rosell, & Benedito, 1999).

For instance CMC has a preferred interaction to proteins while HPMC shows preferential binding to starch (Guarda et al., 2004)

Also taking into account the remarkable heterogeneity of these types of samples, it is very difficult to spot a trend in the behaviour of the considered materials, but an alteration in TG, either a change in slope or a shift to higher or lower temperatures, could suggest that the two hydrocolloids interact differently with the other components of the system, despite leading to similar final effects.

Data interpretation is additionally complicated by the fact that that not only the added hydrocolloids, derived from cellulose, compete for water but also other macromolecules, with molecules such as starches and proteins, with good water binding abilities, influencing the overall result.

3.3.2 Semi-theoretical model for non-isothermal drying kinetics

The water transfer mechanisms in dough systems could be better understood by studying the drying kinetics, even though the drying of materials with high moisture contents is a complicated process, involving simultaneous heat and mass transfer (Yilbas et al. 2003).

Theoretical models need to assume the geometry of a typical food, its mass diffusivity and conductivity (Demirtas et al. 1998); on the other hand, empirical models neglect the fundamentals of drying processes and present a direct relationship between average moisture content and drying time by means of regression analysis (Ozdemir and Devres 1999).

Semi-theoretical models represent a trade-off between the theoretical and empirical ones, derived from simplification of Fick's second law of diffusion or modification of the simplified model, and such models are widely used (e.g. Page, Modified Page, Henderson and Pabis etc.). Results from previous studies, have shown that a simplified 1st order kinetic model can adequately predict moisture loss in dough samples (Fu et al., 2003).

Thermogravimetric analysis (TGA), provides data for that gives an understanding of drying processes (Li, 2005). The weight loss data provided by TGA, and expressed in terms of moisture ratio (MR) versus temperature, have been fitted to semi-theoretical models often used to describe the drying behaviour of agricultural products (Cai and Chen, 2008).

The moisture ratio (MR) can be calculated using the following equation:

$$\pi$$

$$MR = \frac{m_T - m_e}{m_i - m_e} \quad \text{Eq. 3.5}$$

where MR is the moisture ratio, m_T is the mass at temperature T, m_i is the initial mass, and m_e is the mass at the end of the drying process.

All the simplified models for the drying kinetics listed above (e.g. Newton, Page, Modified Page, Henderson and Pabis etc.)., have the same theoretical background summarized below.

It is assumed that mass transfer occurs only by diffusion and diffusive flow can be described according to the Fick's law, the resistance to the moisture flow is uniformly distributed throughout the interior of the homogeneous isotropic material and, if the volume shrinkage is negligible, the resulting mass balance equation, in terms of MR, can be written as follows (Chen et al., 2012)

$$\frac{\partial MR}{\partial t} = \nabla \left[D_{eff} (\nabla MR) \right] \quad \text{(Eq. 3.5a)}$$

The mathematical solution of equation is 3.5a :

$$MR = \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} exp\left(-\frac{(2n+1)^2 \pi^2 D_{eff} t}{4L^2} \right) \quad \text{(Eq. 3.5b)}$$

A simplified version of equation 3.5b can be obtained through a linearization, obtaining

$$\ln(MR) = \ln\left(\frac{8}{\pi^2}\right) - \left(\frac{\pi^2 D_{eff}}{4L^2} t\right) \quad \text{(Eq. 3.5c)}$$

And the MR can be expressed as

$$MR = a \cdot \exp(-kt) \quad \text{(Eq. 3.5d)}$$

Where

$$a = \frac{8}{\pi^2} \quad \text{(Eq. 3.5e)}$$

And

$$k = -\frac{\pi^2 D_{eff}}{4L^2} \quad \text{(Eq. 3.5f)}$$

Equation 3.5d is known as the Henderson and Pabis model.

Newton model is a special case of the Henderson and Pabis Model where the intercept is unity while Page's equation is an empirical modification of Newton model (Akoy, 2014).

The three models considered in this chapter, which are also the most commonly used to describe the isothermal drying processes are listed in Table 3.2.

Model names	Isothermal drying model	
Newton	$MR = \exp(-kt)$	(Eq.3.6)
Henderson and Pabis	$MR = a \cdot \exp(-kt)$	(Eq.3.7)
Page	$MR = \exp(-kt^n)$	(Eq.3.8)

Table 3.2 Isothermal drying models

The derivation of non-isothermal drying models from some isothermal drying analogues has been undertaken in the literature (Hall, 2007), and is presented here.

The parameter k in the models is the drying rate constant, which is dependent upon temperature. The temperature dependence of k may be represented by an Arrhenius-like relationship as follows:

$$k = k_0 \exp\left(-\frac{E}{RT}\right) \quad \text{(Eq.3.9)}$$

where k_0 is the pre-exponential factor, E is the activation energy, R is the universal gas constant, and T is absolute temperature. Under non-isothermal conditions, using a constant heating rate, the relationship between temperature and the time is:

$$T = T_0 + \beta t \qquad \text{(Eq. 3.10)}$$

where T_0 is the initial drying temperature, β is the heating rate and t is the drying time. Substituting Eq.3.9 and 3.10 and into those isothermal models, the corresponding non-isothermal drying models can be obtained and are presented in Table 3.

Model names	Non-isothermal drying model
Newton	$MR = exp\left[-k_0 \exp(-\frac{E}{RT})\frac{T-T_0}{\beta}\right]$ (Eq.3.11)
Henderson and Pabis	$MR = a \cdot exp\left[-k_0 \exp(-\frac{E}{RT})\frac{T-T_0}{\beta}\right]$ (Eq.3.12)
Page	$MR = exp\left[-k_0 \exp(-\frac{E}{RT})\left(\frac{T-T_0}{\beta}\right)^n\right]$ (Eq.3.13)

Table 3.3 Non-isothermal drying models

Fitting to models was evaluated on the basis of the coefficient of determination (R^2) and reduced chi-square (χ^2). In most cases, the R^2 values for the models were greater than the acceptable R^2 value of 0.90, indicating good fits, as shown in Figure 3.12 using the WBF-RC 1.5 sample as an example.
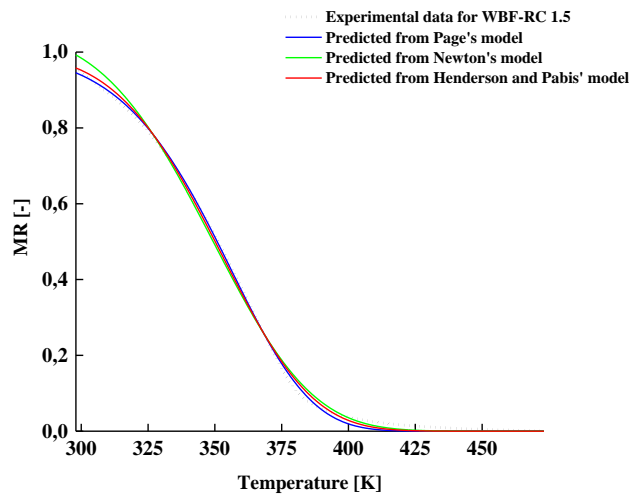
Figure 3.12 Comparison between the experimental and predicted MR

Generally, for all the non-isothermal models, the R^2 value and χ^2 values ranged between 0.9036-0.9999 and 1.2×10^{-4} and 4.6×10^{-4}, respectively. Statistical results for all the samples are collected in Table 3.4.

From Table 3.4, based on the χ^2 *value* and R^2value, Henderson and Pabis' model seems to offer the better compromise to describe the drying characteristics of these kinds of systems.

The Newton model has been excluded because it doesn't fit well the initial stage of the drying process, as it can be also observed from the figure 3.12, while "Henderson and Pabis" and "Page" equations would both be good to describe the phenomenon.

Because for most of the samples the statistic indicators of the best fitting were slighty better in the case of Henderson and Pabis model if compared to the Page's ones, it has been chosen to discuss the results obtained from Henderson and Pabis equation.

The calculated parameter values for the Henderson and Pabis model, for all the samples, are discussed in the section below. When performing TG analysis, samples were tested in triplicate, therefore the value of each parameter and its standard deviation have been calculated as the mean value of the three fittings performed for each sample.

Sample ID	Page		Newton		Henderson and Pabis	
	R^2 value (avg)	χ^2 value	R^2 value (avg)	χ^2 value	R^2 value (avg)	χ^2 value
WF	0.9036	1.6E-02	0.9924	1.2E-03	0.9962	6.2E-04
WBF	0.9990	1.6E-04	0.9976	3.7E-04	0.9998	2.0E-04
R	0.9987	2.0E-04	0.9978	3.4E-04	0.9989	1.7E-04
WBF-R	0.9992	1.3E-04	0.9982	2.8E-04	0.9998	1.5E-04
WBF-RH 0.1	0.9992	1.3E-04	0.9980	3.2E-04	0.9991	1.4E-04
WBF-RH 0.5	0.9987	2.0E-04	0.9976	3.9E-04	0.9989	2.0E-04
WBF-RH1	0.9989	1.7E-04	0.9974	4.1E-04	0.9989	2.0E-04
WBF-RH 1.5	0.9990	1.6E-04	0.9974	4.1E-04	0.9988	1.9E-04
WBF-RH85%W	0.9987	2.1E-04	0.9975	4.1E-04	0.9987	2.1E-04
WBF-RH100%W	0.9987	2.2E-04	0.9972	4.6E-04	0.9985	2.5E-04
WBF-RC 0.1	0.9989	1.7E-04	0.9976	3.8E-04	0.9988	1.9E-04
WBF-RC 0.5	0.9990	1.6E-04	0.9982	2.6E-04	0.9990	1.5E-04
WBF-RC 1	0.9989	1.6E-04	0.9979	3.2E-04	0.9989	1.7E-04
WBF-RC 1.5	0.9989	1.7E-04	0.9976	3.6E-04	0.9999	1.7E-04
WBF-RC85%W	0.9991	1.4E-04	0.9979	3.2E-04	0.9999	1.7E-04
WBF-RH100%W	0.9985	2.4E-04	0.9973	4.3E-04	0.9987	2.4E-04

Table 3.4 Statistical results from non-isothermal drying models

3.3.2.1 Results for control breads with different flours

Figures 3.13 and 3.14 shows clear differences for the Henderson and Pabis model parameters, for the gluten and gluten free dough. The pre-exponential factor k_0 and the activation energy E vary, dependent on the type of dough. Both k_0 and E values for the GF samples are lower than for the WF doughs. For WF the activation energy is ~ 44 kJ/mol, while for both gluten free doughs (WFB, R) E is ~ 30 kJ/mol; WBF and R parameters are comparable, with a higher standard deviation on the R sample. These energies could indicate that more energy is required to remove water from the dough containing gluten.

Despite the noticeable differences between the two types of dough, the activation energies found for all the samples are in good agreement with literature values for other food materials (Ndapeu et al., 2013; Thaeri-Garavand et al., 2011; Fu et al., 2003; Roberts and Tong, 2003).
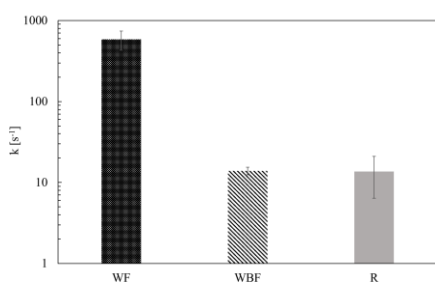
Figure 3.13 - Calculated k_0 parameter values of Henderson and Pabis model for WF, WBF and R samples
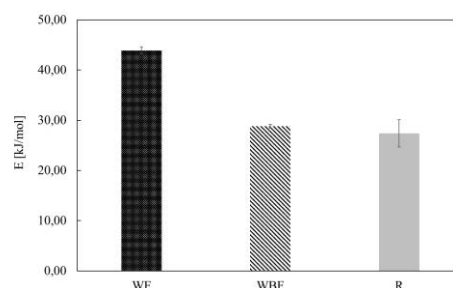
Figure 3.14 - Calculated E parameter values of Henderson and Pabis model for WF, WBF and R samples

No differences were observed for the value of the 'a' fitting parameter, as shown in Figure 3.15.
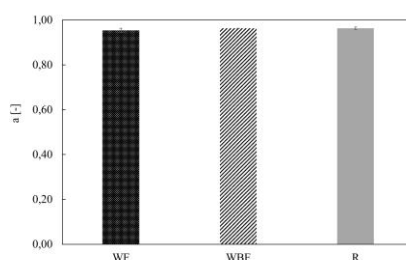
Figure 3.15 - Calculated a parameter values of Henderson and Pabis model for WF, WBF and R samples

3.3.2.2 Results for breads with different hydrocolloid and water concentration

Effects related to the variation of the amount of hydrocolloid added to the dough mix can also be discussed by evaluating the activation energy trend for the analysed materials. Samples with some level of HPMC exhibit higher E values than the control bread at all hydrocolloid

concentrations, whereas, for CMC, with the exception of the WBF-RC0.1 sample, all other samples exhibit E values lower than or equal to that of the control bread.

Furthermore, both for CMC and HPMC, a similar trend seems to be observed with increasing concentration of hydrocolloid. At 0.5%, there is a decrease in the activation energy, especially in the case of the sample with CMC. At higher concentrations, the E parameter increases again, reaching a value that no longer changes considerably
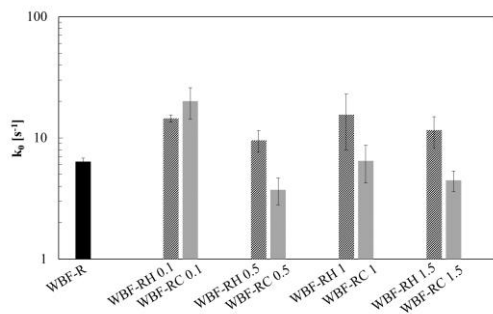
Figure 3.16 Calculated k_0 parameter values of Henderson and Pabis model for WBF-R control and for samples prepared with CMC and HPMC at different concentration
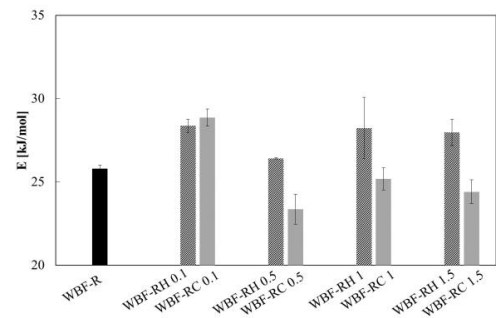
Figure 3.17 Calculated E parameter values of Henderson and Pabis model for WBF-R control and for samples prepared with CMC and HPMC at different concentration

Considering the results obtained for the samples with different water concentrations, the E values for the HPMC samples are again higher than for CMC. A similar trend is also observed for both types of water binder.
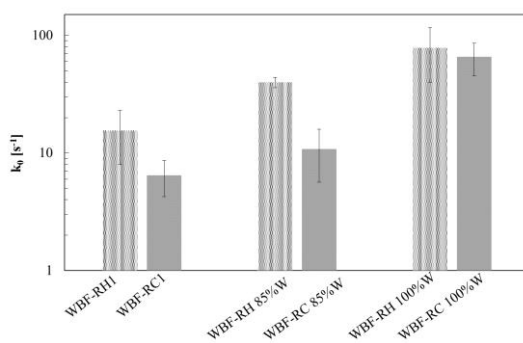
Figure 3.18 - Calculated k_0 parameter values of Henderson and Pabis model for samples prepared with CMC and HPMC at 1% and different water concentration
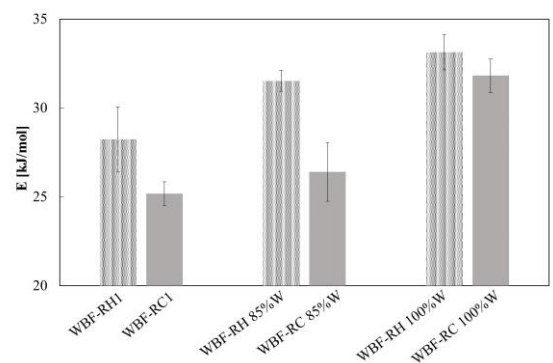
Figure 3.19 - Calculated E parameter values of Henderson and Pabis model for for samples prepared with CMC and HPMC at 1% and different water concentration

When increasing the concentration of water, it is notable that the E value, obtained for the sample with the highest water content, is close to that observed for the sample containing gluten,

however, as for all GF samples, it is still below that value. At the same time, an increase in water content of the doughs results in an increase in loaf volume of the GF bread, as confirmed by the empirical baking tests discussed in Chapter 2.

Since proteins and polysaccharides are the main construction materials of food, their interactions with water, and with each other in the water medium, are known to govern structure–property relationships in foodstuffs (Matveev et al., 2000).

Significant improvements in the honeycombed structure are actually observed only by increasing the water content in the GF dough.

No interesting improvements are observed when changing the hydrocolloid concentration, suggesting that the presence of a quantity of water over 70% (on a flour basis) in the studied systems, could improve the interactions between the biopolymers (non glutenous-proteins naturally present in WBF, and non-starch-polysaccharides added as CMC and HPM, but also from starch naturally contained in the buckwheat and rice flour) present in the dough by promoting the formation of a stronger network, capable of entrapping the carbon dioxide produced during yeast fermentation, and allowing the bread to expand its volume.

Both proteins and polysaccharides can contribute to the structural and textural properties of foods through their aggregation and gelation behaviour (Patino and Pilosof, 2011), even if the mechanism involved in the dough formation is not yet clear. Protein and polysaccharide molecules can link together by covalent bonds, giving a specific, strong and essentially permanent 'conjugate', or they can associate via non-covalent interactions (electrostatic and hydrophobic interactions, steric exclusion, hydrogen bonding, etc.) between the biopolymers, influencing the interfacial characteristics of adsorbed films and the formation and stability of the dispersion (Patino and Pilosof, 2011).

The foam structure of a fermenting dough has been assumed to be a dispersion of discrete gas cells in a continuous polysaccharide–protein matrix (Hayman et al., 1998), therefore, surface tension in the gas-dough interface is one factor that may affect the behaviour of gas cells dispersed in the continuous dough phase (Hayman et al., 1998).

Again, no differences were observed for the value of the 'a' fitting parameter, as shown in Figure 3.20, which, in this case, as well as for control doughs, is again ~ 0.96.
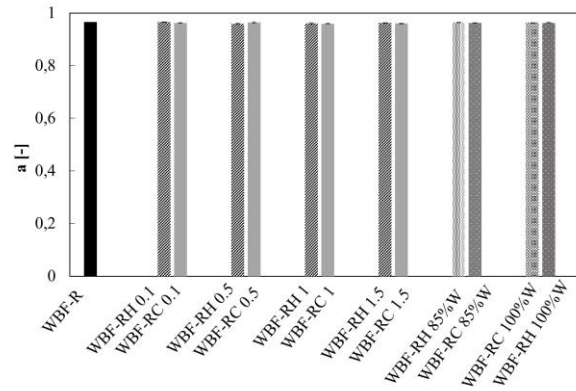
Figure 3.20 Calculated a parameter values of Henderson and Pabis model for all the analysed samples, at different hydrocolloid (CMC and HPMC) concentration and at different water content

3.3.3 DTG trace analysis

A more direct impact of the dehydration phenomenon and of the characteristic temperatures of the process, can be observed from the trace of the time derivative, DTG (derivative of thermogravimetry), which shows a better-defined peak the maximum of which corresponds to the flexus point in the TG trace. From the plot in Figure 3.21, it can be seen that the GF doughs exhibits peak water loss at lower temperatures when compared to the wheat flour dough, as already discussed in Section 3.3.1. While for the WBF and R samples, the peak can be observed around 100 °C and 85 °C, respectively, for the wheat flour the peak it is around 125 °C.
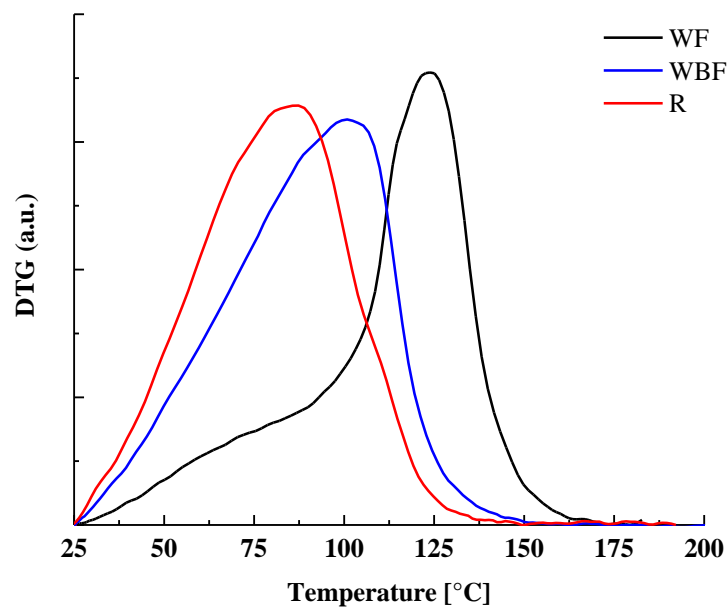
Figure 3.21 – DTG traces for WF, WBF and R samples

All the analysed GF samples showed behaviour similar to that observed for WF and R samples. Mixing rice and buckwheat, the sample (WBF-R) exhibited peak water loss at an intermediate temperature, between those for WBF and R samples, around 93 °C.
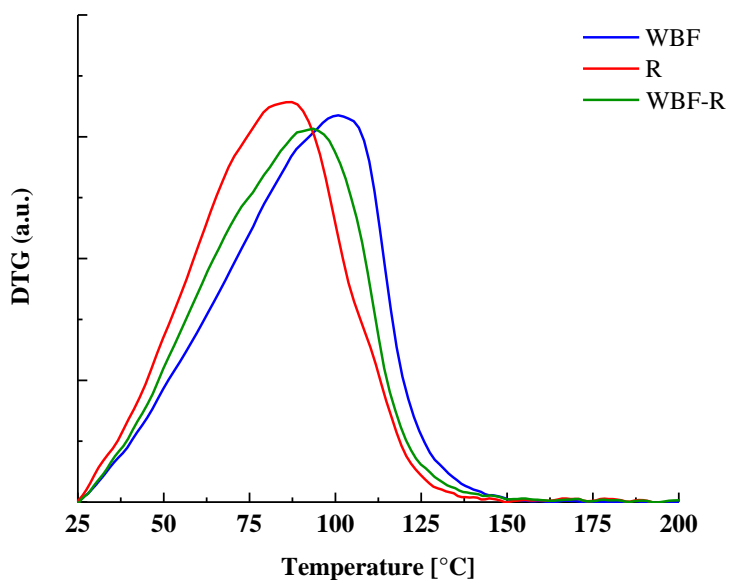
Figure 3.22 - DTG trace for WBF, WBF-R and R samples

The DTG traces for the samples prepared using HPMC are given in Figures 3.23 and 3.24.
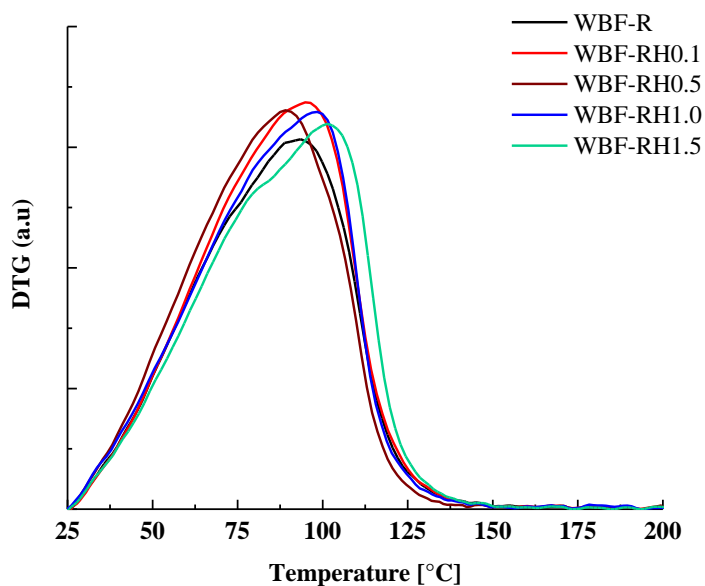
Figure 3.23 - DTG traces for WBF-R, WBF-RH0.1, WBF-RH0.5, WBF-RH1, WBF-RH1.5 samples
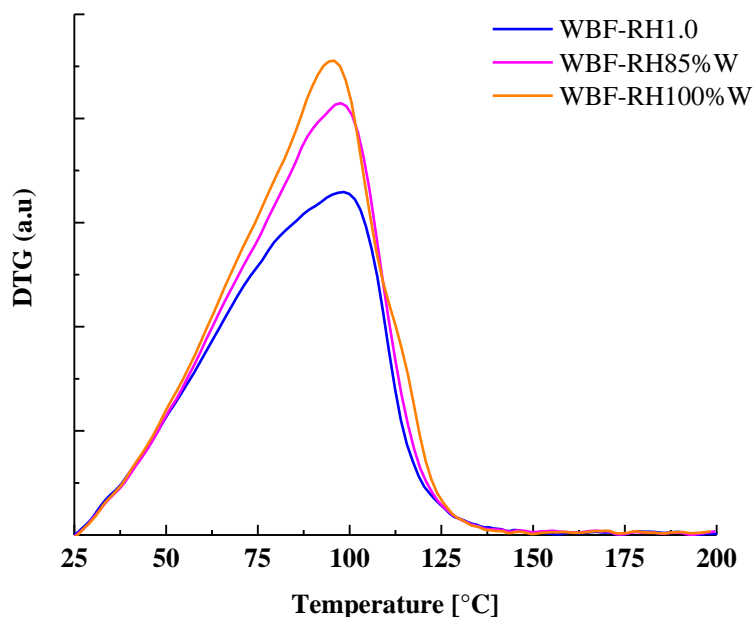
Figure 3.24 - DTG traces recorded for WBF-RH1, WBF-RH85%W and WBF-RH100%W samples

The DTG traces provide observation that an increase in the hydrocolloid amount seems to determine a forward shift in the maximum, to higher temperatures (Figure 3.23), while increasing the amount of water leads to a sharper and higher peak (Figure 3.24).

The DTG traces for the samples prepared using CMC are reported in Figure 3.25. CMC samples exhibit a similar behaviour when compared to the HPMC series, in terms of the slight shift observed in temperature corresponding to the maximum high of the DTG trace (T_{peak}).
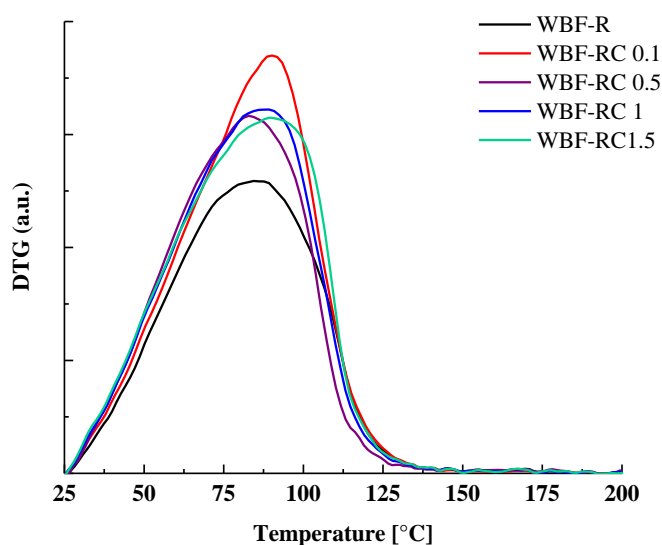
Figure 3.25 - DTG traces for WBF-R, WBF-RC0.1, WBF-RC0.5, WBF-RC1 and WBF-RC1.5 samples

In addition, in the case of samples with different water contents, also for the CMC hydrocolloid, increasing the water percentage led to a sharper and higher peak, as shown in Figure 3.26.

The trend shows that the Tpeak for both CMC and HPMC samples is not affected by the water percentage. The increase in the peak high (i.e. the rate of the mass loss) and area with increasing the moisture content is simply due to the differing quantity of water contained in the samples. The area underneath the curve represents in fact the quantity of evaporated water which is higher in the WBF-RH100%.
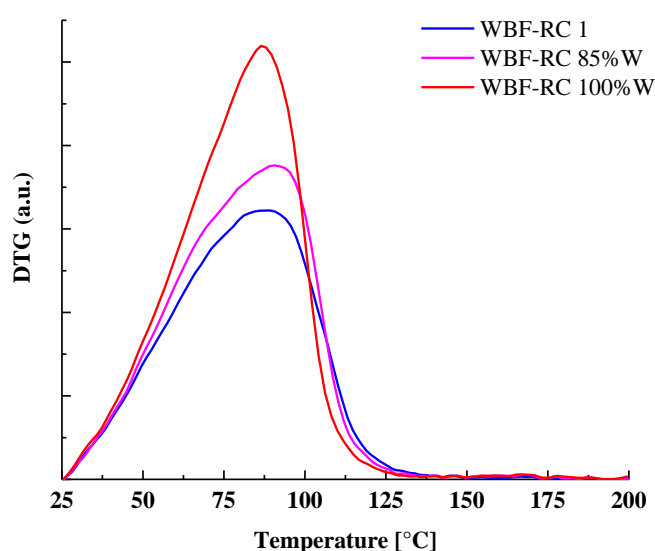
Figure 3.26   DTG traces for WBF-RC1, WBF-RC85%W and WBF-RC100%W samples

3.3.4 DTG trace deconvolution

The enthalpies of the different water fractions, present in the food matrix under different conditions (either because of different molecular mobility or because of tertiary force links of different strength with the substrate of a given food system), are some order of magnitude smaller than the vaporization enthalpy. This means that it is not often possible to clearly distinguish between the different enthalpies in the DSC trace. However, different states of water can be recognised, as a consequence of the different temperatures at which water is released during an experimental run, performing deconvolution of the broad DTG, thereby splitting it into the main Gaussian components, each relevant to a fraction of dough moisture (Fessas and Schiraldi 2001). An approximate picture can be obtained by resolving the DTG (or DSC) signal into two or three main Gaussian components. The deconvolution of the DSC signal would,

consequentially, give the enthalpy associated with the vaporization of the corresponding single water fraction.

The water fraction released at lower temperature is more mobile, evaporating at mild temperatures, and belongs to the imbibing moisture (in the earlier steps) and to the aqueous phase that separates because of the thermodynamic incompatibility of the solutes (such as carbohydrates and proteins). The rest of the water tends to remain within the network, forming polymers, and can be stripped away only at higher temperatures.

Before analysing the GF samples, deconvolution of the wheat flour dough DTG trace was performed. The DTG plot for dehydration of the WF glutenous sample was resolved into two main Gaussian components, as shown in Figure 3.27. According to previous studies (Schiraldi et al., 2003), each component is relevant to a fraction of the dough moisture content.

One fraction (Water II) is released above 100°C (around 124°C ± 3), which could be referred to as 'structural water', i.e. water that is part of the structure of the substrate.

Water is ubiquitous in food products, where, because of its small molecular mass, is the major mobile component. As it easily forms hydrogen bonds with a number of substrates, water can either solvate ions and/or polar molecules (or functional groups) keeping them apart from one another, or become a structure component of supra-molecular clusters. In the case of doughs, the Water II fraction is therefore to be considered the more physically entrapped water, the held water associated to starch faction and proteins undergoing reticulation. This fraction is hard to remove and does not contribute to the overall relative humidity (RH) of the system.

In the case of wheat flour dough, structural water is mainly linked to the gluten component.

The rest of the dough moisture (Water I) is instead released around 97°C ± 3.3. This should be the water released by simple diffusion, free to evaporate (with a fickian diffusion law from the core to the surface of the dough). This water fraction released at lower temperature is more mobile and has an easier access to the head space of the sample; the corresponding aqueous phase, that wets the starch granules and contains most of water soluble solutes (salts, simple sugars, globular proteins, etc.) and mainly plays a plasticizing role, is more "directly" involved in any determination of the sample RH.
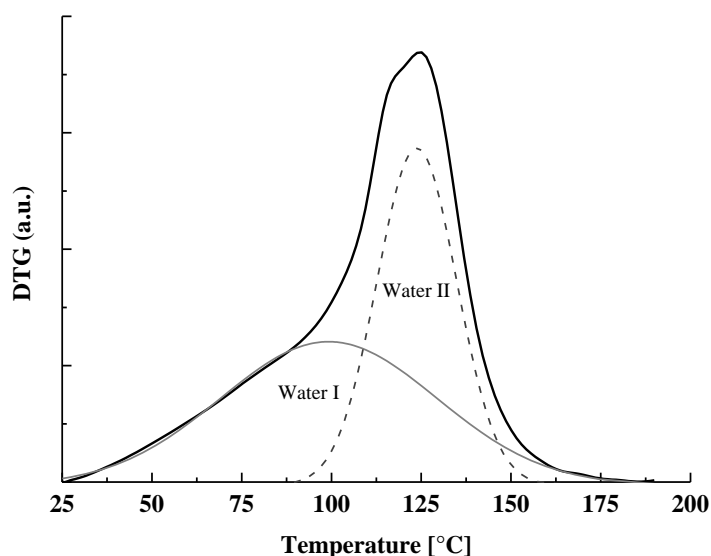
Figure 3.27 – Deconvolution of the DTG trace for the WF sample, resolved into two Gaussian components

When discussing these types of data, some considerations must be taken into account. For instance, the starting conditions of the system strongly influence the release of water occurring during the TG run, while no important effects come from changes in the system structure. This means that any treatment, like mixing and kneading at room temperature, can, therefore, affect water partitioning within the dough (the amount of water in each fraction) in advance of the experimental run; the relevant T_{vap} determines changes in the DTG trace, while no effects come from starch gelatinization and gluten reticulation.

The results obtained for deconvolution of the WBF-R sample are reported, as an example, in Figure 3.28. The shape of the DTG differs from that of the WF sample, but also in this case a broad peak is obtained, in agreement with previous work on GF doughs (Crockett et al., 2011)

The sample dehydration, described by a single broad peak of the DTG trace, appears to be a single process controlled by the moisture diffusion from the core to the surface of the material. According to Fessas and Schiraldi 2008, this should mean that the starch polymers and the non-gluten proteins do not really compete for water. According to this assumption, it seems that because carbohydrates and proteins could be thermodynamically incompatible, the separated aqueous phases could easily exchange the solvent between one another therefore the water evaporating from one aqueous phase is rapidly substituted by the water moving from any other nearby aqueous phase.
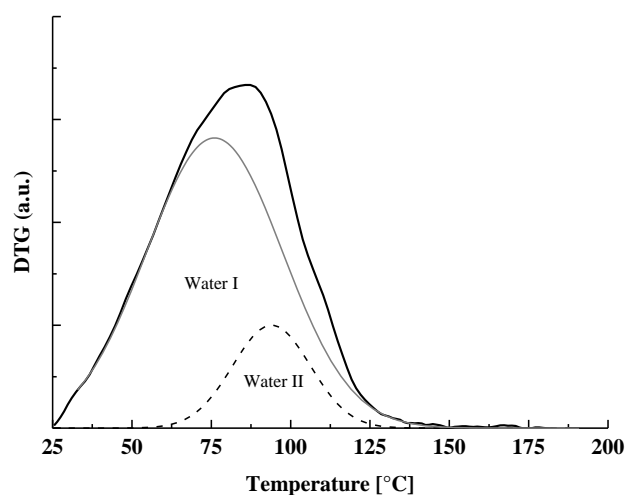
Figure 3.28 Deconvolution of the DTG trace for the gluten free WBF-R sample into a couple
of Gaussian components

Deconvolution of the DTG trace was performed on all samples, prepared with CMC and HPMC, and the results are reported in terms of average water contribution and average peak temperatures (Table 3.2).

Sample ID	Average water contribution [%]		Average Peak Temperature [˚C]	
	Water 1	Water 2	T_{peak} 1	T_{peak} 2
WF	50 ± 3	50 ± 3	97 ± 2	124 ± 1
R	80 ± 1	20 ± 1	75 ± 5	95 ± 7
WBF	75.7 ± 0.4	24.3 ± 0.4	82.4 ± 0.2	104.4 ± 0.6
WBF-R	80 ± 8	20 ± 8	74.7 ± 3.4	94.9 ± 7.0
WBF-RH 0.1	80.2 ± 0.5	19.8 ± 0.5	79.9 ± 2.2	100.2 ± 2.1
WBF-RH 0.5	78.1 ± 2.3	21.9 ± 2.3	76.4 ± 3.8	96.8 ± 5.8
WBF-RH 1.0	77.9 ± 2.5	22.1 ± 2.5	78.6 ± 0.6	100.3 ± 1.4
WBF-RH 1.5	71.1 ± 7.2	28.9 ± 7.2	78.7 ± 3	104.3 ± 2.5
WBF-RH 85%W	71.3 ± 4.8	28.7 ± 4.8	78.5 ± 0.4	99.4 ± 1.6
WBF-RH 100%W	68 ± 5	32 ± 5	78 ± 1	99.1 ± 4.6
WBF-RC 0.1	76.8 ± 0.7	23.2 ± 0.7	76.7 ± 2	95.1 ± 3.6
WBF-RC 0.5	53.3 ± 7.6	46.7 ± 7.6	65.7 ± 2.2	91.4 ± 1.5
WBF-RC 1.0	80.9 ± 2.4	19.1 ± 2.4	75.1 ± 1.4	96 ± 4
WBF-RC 1.5	79.4 ± 0.7	20.6 ± 0.7	75.1 ± 0.9	98.7 ± 1.2
WBF-RC 85%W	78.6 ± 0.7	21.4 ± 0.7	74.6 ± 0.3	95.9 ± 0.2
WBF-RC 100%W	70.2 ± 1.2	29.8 ± 1.2	72.4 ± 0.7	90.3 ± 0.8

Table 3.2 - Water peak distribution and peak temperature of the deconvoluted DTG curves

The main differences in these systems can be observed between the two types of dough. The evaporation of the most tightly bound water in the GF dough occurred at about 95 to 100 ˚C, below that of the water loss for WF dough, which occurs around 125˚C. For all the GF samples, the area for the "water peak" was the largest, representing the majority of the water evaporated, with peak temperatures ranging from 66˚C to 82˚C.

DTG curve deconvolution of the hydrocolloid-added formulations showed some differences in terms of T_{peak} and water contribution. For the HPMC samples, increasing the amount of hydrocolloid slightly increased the Water 2 peak area from $19.8 \pm 0.5\%$ to $28.9 \pm 7.2\%$, and the T_{peak2} from 100.2 ± 1.4 ˚C to 104.3 ± 2.5 ˚C.

Changing the amount of water also slightly increased the Water 2 peak area from $22 \pm 2.5\%$ to $32 \pm 4.9\%$, while no relevant differences were observed in T_{peak2}, which ranged from $100 \pm 2.1°C$ to $99 \pm 4.6°$ C.

A different behaviour was observed for the CMC samples. The Water 2 peak area decreased going from 0.1% to 0.5% of CMC, before increasing again to an average of 20% for higher hydrocolloid concentrations. T_{peak2} increased slightly, from $95.1 \pm 3.6°$ C to $98.7 \pm 1.2°$ C, with increasing the CMC amount. In changing the water amount in the CMC samples, the Water 2 peak area increased from $19.1 \pm 2.4\%$ to $29.8 \pm 1.2\%$, while T_{peak2} decreased from $96 \pm 4°$ C to $90.3 \pm 0.8°$ C.

The most interesting result here is that T_{peak2} for the HPMC samples were higher than T_{peak2} found for CMC samples, leading to the conclusion that HPMC exhibits better water retention abilities at higher temperatures than CMC, and a more regular behaviour with increasing hydrocolloid concentration.

3.3.5 Spectroscopic Analysis

Mid-infrared spectroscopy in food analysis

Some of the characteristic absorption bands in the M-IR range, associated with major components of food, which will be useful in interpreting the experimental results, are discussed below. Water bands are present at 3920, 3490, 3280, and 1645 cm^{-1}, although the exact location and shape of these bands may be affected by the presence of solutes (Lin and Brown, 1992), hydrogen bonding and temperature (Libnau et a., 1994). The triglyceride ester linkage C-O at~1175 cm^{-1}, the C=O group (~1750 cm^{-1}), and the acyl chain C-H (3000-2800 cm^{-1}) frequencies are commonly used to determine fats (Ripoche and Guillard, 2001), while the amide

I ($\sim$1653 cm^{-1}) and II bands ($\sim$1567 cm^{-1}) have been used for the estimation of proteins and changes in protein secondary structure (Dousseau and Pezolet, 1990). Vibrations arising from C-O and C-H stretches in the region between 1100 and 1000 cm^{-1} may be used to identify aqueous sugar molecules (Hashimoto and Kameoka, 2008), while more complex carbohydrate structures, found in plants, have major absorption bands at higher wavenumbers, for example, hemicellulose (1732, 1240 cm^{-1}), cellulose (1170-1150, 1050, 1030 cm^{-1}), lignin (1590, 1510 cm^{-1}), and pectin (1680-1600, 1260, 955 cm^{-1}) (Michell and Schimleck, 1996). Absorption in the fingerprint region is mainly caused by bending and skeletal vibrations (Karoui et al., 2010).

3.3.5.1 FT-IR spectra for bread ingredients

FTIR spectra for the gluten free flours used in this study are reported in Figure 3.29.
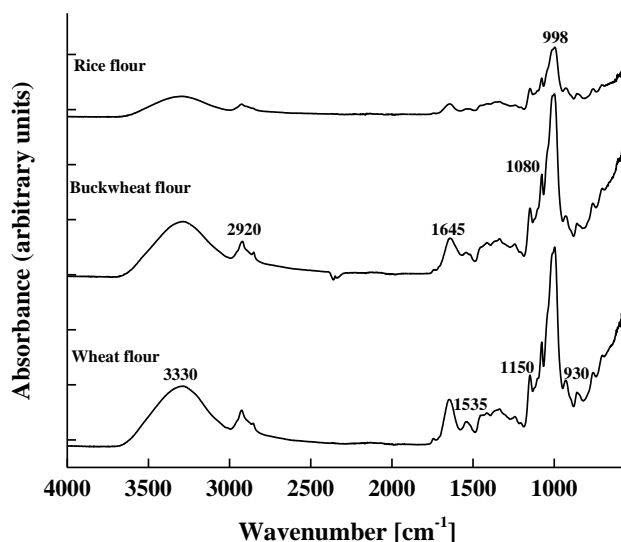
Figure 3.29 - Normalised FTIR spectra for ingredients used for bread making.

All powders were analysed in the dry state after heating at 40° C for 24 h to remove any excess moisture. All ingredients, showed bands at 2920-3000 cm^{-1}, due to C-H stretching modes, and a broad peak at 3280-3300 cm^{-1}, due to intermolecular H-bonding and the O-H stretching mode of water (Sivam et al. 2013, Sinelli, Casiraghi and Downey 2008). The two bands around 1643 and 1535 cm^{-1} are mainly associated with flour proteins and are assigned to Amides I and II, respectively. These bands are very weak, or even absent, for the rice flour sample, which is, in fact, poor in proteins. The analysed samples have absorption bands at 900–1200 cm^{-1} (i), which are characteristic of polysaccharides (Sivam et al. 2013, Cerna et al. 2003,

Kacurakova et al. 2000). Bands at 1080 and 1150 cm^{-1} are associated with the coupled C-O and C-C stretching vibrations of polysaccharide molecules.

Spectra for the hydrocolloids used in the formulation are shown in Figure 3.30.
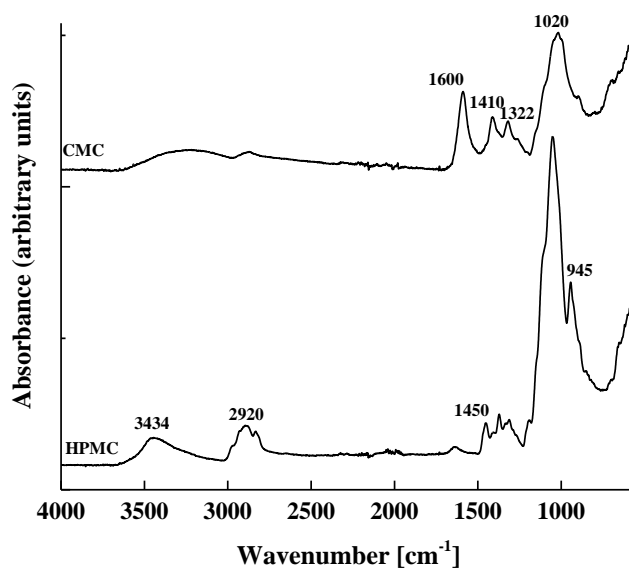
Figure 3.30 - Normalised FTIR spectra for pure hydrocolloid powders added in the GF bread formulations

The stretching frequency of the cellulose –OH group appears around 3434 cm^{-1} (Huang et al., 2010). Also for the analysed cellulose derivative hydrocolloids, the band at 2920 cm^{-1}, due to C–H stretching vibration, can be observed and the absorption band around 1600 cm^{-1} is assigned to the stretching vibration of the carboxyl group (COO$^-$) (Pushpamalar et al., 2006). The bands around 1416 and 1322 cm^{-1} are related to the -CH$_2$ scissoring and -OH bending vibrations, respectively (Pushpamalar et al., 2006); while the complex band between 1220 and 950 cm^{-1} (associated with the cellulose polymer) comprises numerous C–O vibrations, including glycosidic C–O–C, C–OH, C–OCH$_3$, C–OCH$_2$CH$_2$OH, (Bajwa et al., 2009). Band assignments referred to HPMC and CMC used in the present work, are based on those reported for cellulose and some of its derivatives (Sammon et al., 2006)

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3.3.5.2 FT-IR for control and treated breads

The FTIR spectra in the full investigated wavenumber range, for all the samples, are reported below in Figures 3.31-3.33.
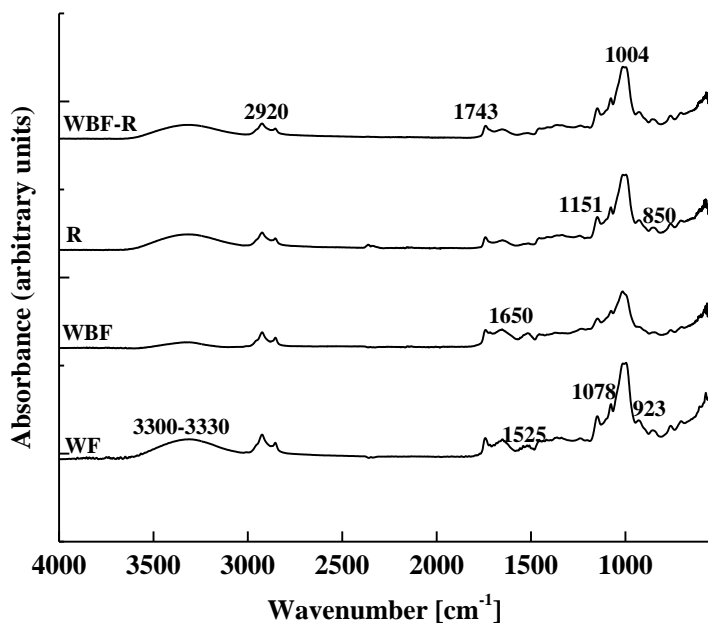
Figure 3.31 - Normalised FTIR spectra for control breads (WF, WBF, R and WBF-R)
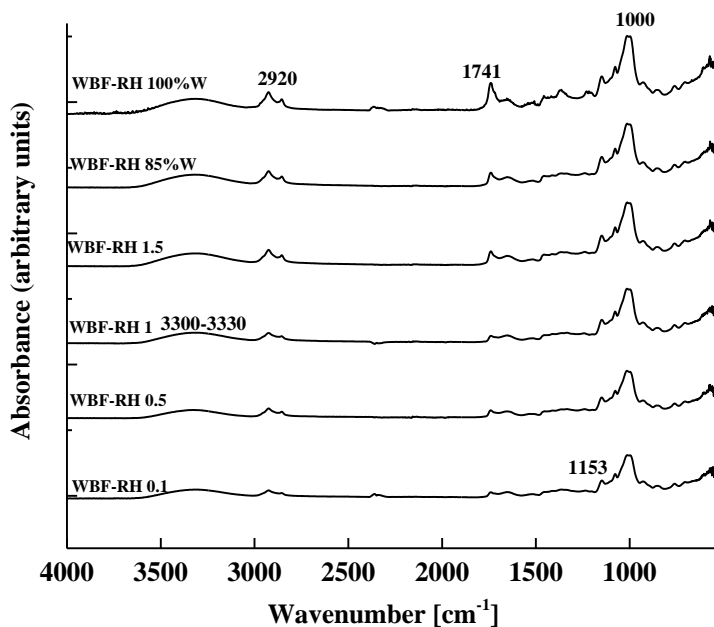
Figure 3.32 - Normalised FTIR spectra for samples added with HPMC
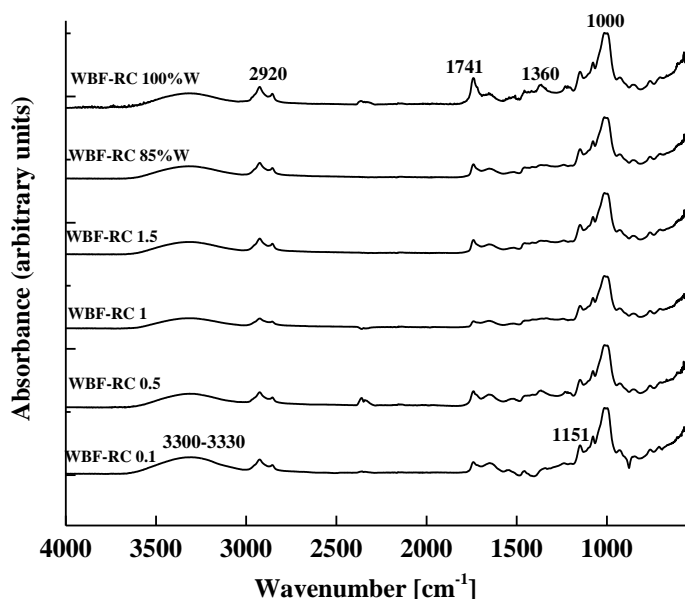
Figure 3.33 - Normalised FTIR spectra for samples added with CMC

All samples exhibited similar spectra; the evident peaks observed in all the analysed samples, are due to the main component of the dough, as water, starch, proteins and fat. The peak at 3300 cm^{-1} originates from OH and N-H bond presence (Robertson et al., 2006). Bands at 2920-3000 cm^{-1}, are due to C-H stretching modes, and the band near 1000 cm^{-1} is due to the intramolecular hydrogen bonding of hydroxyl groups. The peak at 1745-1747 cm^{-1} belongs to the carbonyl stretching frequency of the fatty ester groups from lipids (van Velzen et al. 2003). While the region from 1500-1600 cm^{-1} is called amide II, which is primarily due to N-H bending C-N stretching and it is particularly sensitive to protein-solvent interaction and protein-protein hydrophobic interaction (Mejia, Mauer and Hamaker 2007).

3.3.5.3 Amide I and amide II bands analysis

The deconvoluted amide I and amide II regions, for all analysed samples, consist of overlapping component bands, representing α-helices, β-sheets, turns and random structures. For both the control samples (Figure 3.34-37) and those with added hydrocolloids (Figures 3.38-3.49), the amide I band is centred around 1650, exhibiting an asymmetrical shape, and the amide II band is centred around 1520 cm^{-1}. Considerable variations in the line shapes of these bands can be observed among the studied samples. All samples also exhibit a peak around 1742 cm^{-1}, which is due to the carbonyl group stretching of ester groups. The intensity of this peak changes between samples.

Control doughs

The deconvoluted amide I region for the WF dough showed a prominent band around 1641 cm^{-1} (s). Bands in the regions of 1620−1640 cm^{-1} and 1695−1690 cm^{-1} can be assigned to β-sheet conformation, as previously reported (Barth, 2007; Kong and Yu 2007). A minor band can be observed around 1651 cm^{-1} (w); components centred between approximately 1658 and 1650 cm^{-1} have been assigned to the α-helix (Kong and Yu 2007, Nevskaya and Chirgadze, 1976). Another peak around 1674 cm^{-1} (m) (β-turns) is observed. Bands around 1670, 1683, 1688 and 1694 cm^{-1} are usually assigned to β-turns (Kong and Yu 2007, Barth 2007) and are known, on an experimental basis, to give rise to the amide I bands in a wide wave number range (1660-1695 cm^{-1}), but this often overlaps with the higher wavenumber band of β-sheets, making the assignments difficult (Torii and Tasumi, 1992). Peaks at 1522, 1541 and 1558 cm^{-1}(β-turns) were deconvoluted from the amide II region and can be ascribed to secondary amide bending modes, while peaks around 1507 and 1493 cm^{-1} can be assigned to β-sheets (extended and intermolecular bonding) (Sivam et al., 2013).
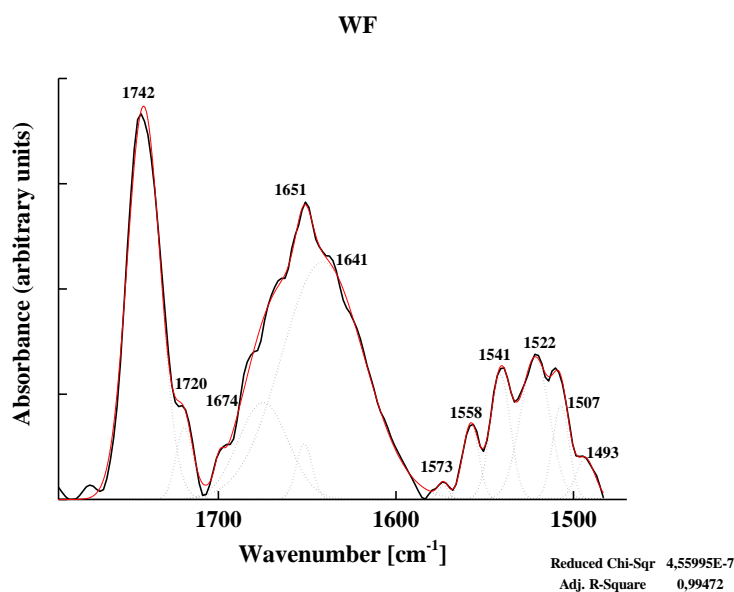
Figure 3.34 - FTIR spectra in the 1800–1450 cm^{-1} range for WF dough sample

The overall signal of the amide I band is attenuated in the GF control breads. WBF band deconvolution led to similar sized peaks at 1657 cm^{-1} (m) and 1629 cm^{-1} (m), typical of β-turns and β-sheets that are strongly H-bonded (Byler and Susi, 1986). Weaker peaks, around 1678 cm^{-1} (w) (β-turns) and 1601 cm^{-1}, are probably due to side chain vibrations.
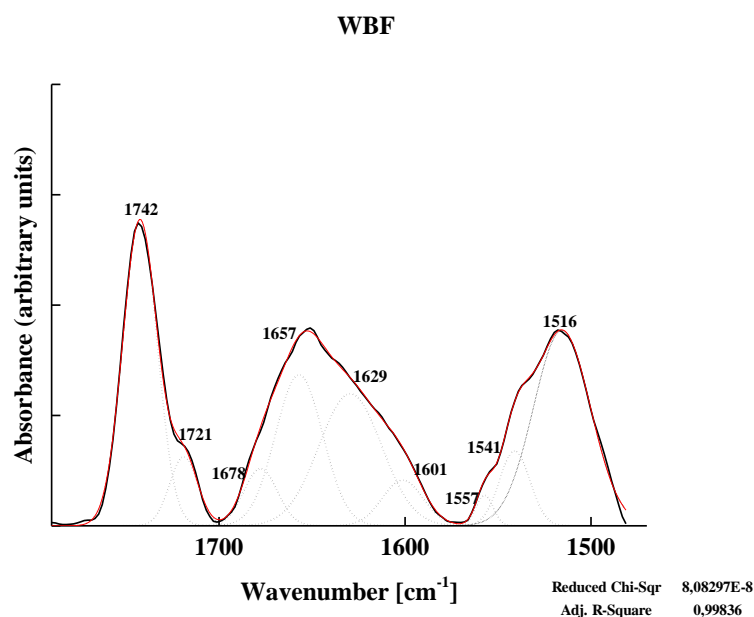
Figure 3.35 - FTIR spectra in the 1800–1450 cm⁻¹ range for WBF dough sample

In the R and WBF-R samples (Figures 3.36-3.27), a further reduction of the amide II band is observed. The poor protein content of the rice flour seems to reflects on the IR amide I band of the R samples which lack in β-sheets, exhibiting only bands assignable to β-turns (1680 cm⁻¹ (w) and 1658 cm⁻¹ (w)) and α-helix (1635 cm⁻¹ (m))

R
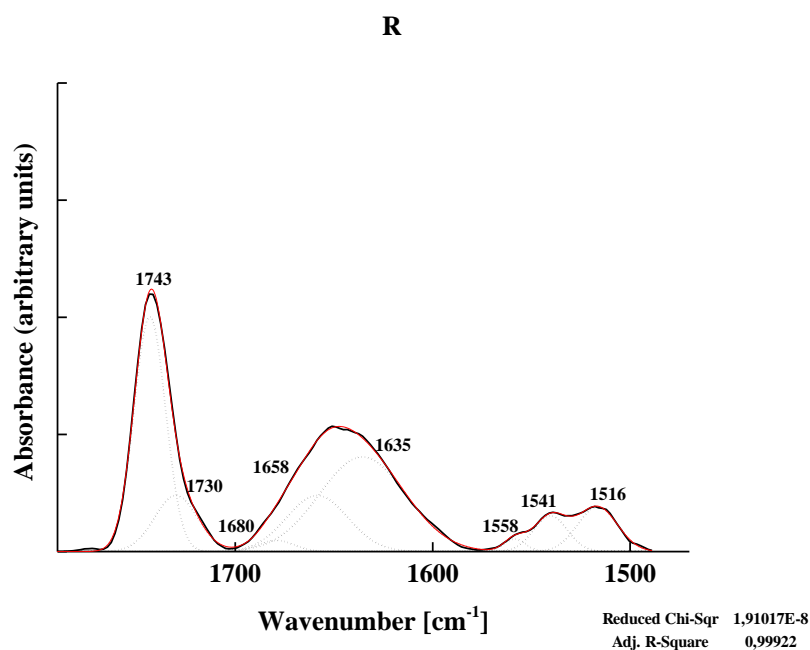
Figure 3.36 FTIR spectra in the 1800–1450 cm⁻¹ range for R dough sample

In the WBF-R sample, which represent the control GF bread chosen as a promising starting point for hydrocolloid addition, strongly H-bonded β-sheets (1626 cm⁻¹ (w)) probably due to

the protein contribution from the buckwheat flour, α-helix (1646 cm^{-1} (m)) and β-turns (1667 cm^{-1}) are observed.
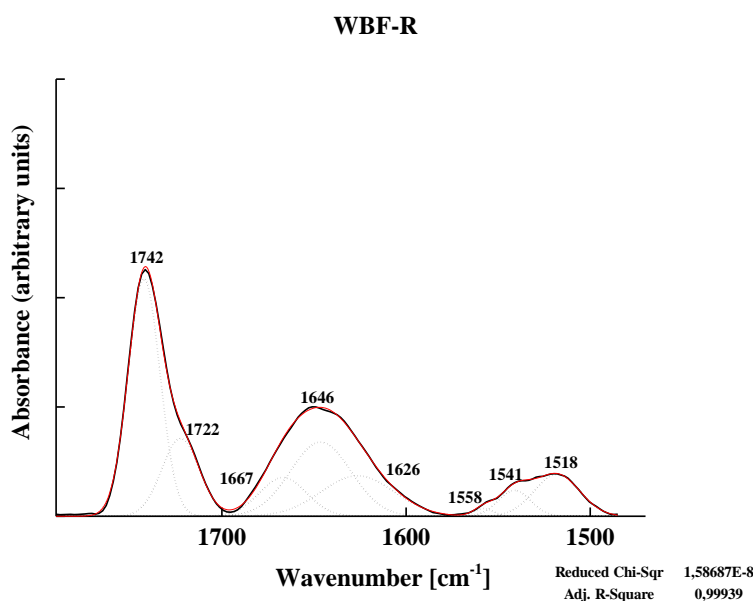
Figure 3.37 - FTIR spectra in the 1800–1450 cm^{-1} range for WBF-R dough sample

Effect of HPMC addition

In the WBF-R sample (Figure 3.37) the dominant peak is the one around 1646 cm^{-1}, assigned to the α-helix conformation. Further peaks, around 1626 cm^{-1} (β-sheet strongly bonded) and another at 1667 cm^{-1} (β-turn), are also observed.

Adding hydrocolloid did not significantly affect the overall intensity of the amide I and amide II bands, but resulted in a progressive reduction and then the disappearance of the α-helices peak (1655 cm^{-1} (Figure 3.38) and 1652 cm^{-1} (Figure 3.39)) and an increasing presence of β-sheets conformations (1641 cm^{-1} assigned to β-sheet weakly bonded (Figure 3.38), 1624 cm^{-1} assigned to β-sheet strongly bonded (Figure 3.39), 1640 cm^{-1} β-sheet weakly bonded (Figure 3.39), 1638 cm^{-1} assigned to β-sheet weakly bonded (Figure 3.40), 1638 cm^{-1} assigned to β-sheet weakly bonded (Figure 3.41)).

Interactions between flour proteins and polysaccharides has been studied and their effects on protein secondary structure were investigated by FT-IR spectroscopy in previous works (Secundo and Guerrieri, 2005). It was observed that a decrease in α-helices, and slightly more intermolecular H-bonding conformational changes in the amide I band, were induced by the addition of other polysaccharides, which compete with flour proteins for water (Sivam et al., 2012). This was also observed in the studied samples, suggesting that the added cellulose derivative hydrocolloids could form a complex with the natural protein contained in the flour, thereby affecting the profile of the amide I and amide II bands.

In previous studies, it has been stated that proteins and polysaccharides mixed in aqueous media, could form hybrids (complexes) with enhanced functional properties in comparison to proteins and polysaccharides alone (Benichou et al., 2010).
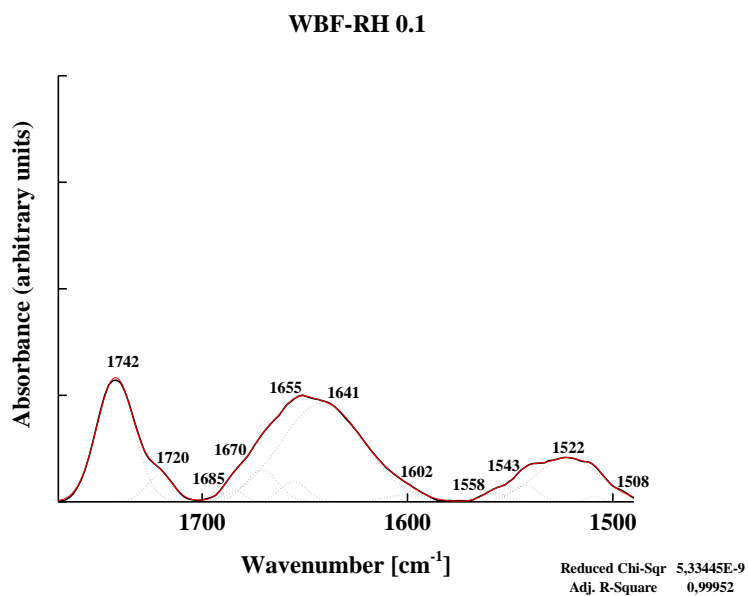
WBF-RH 0.1

Figure 3.38 - FTIR spectra in the 1800–1450 cm^{-1} range for WBF-RH0.1 dough sample
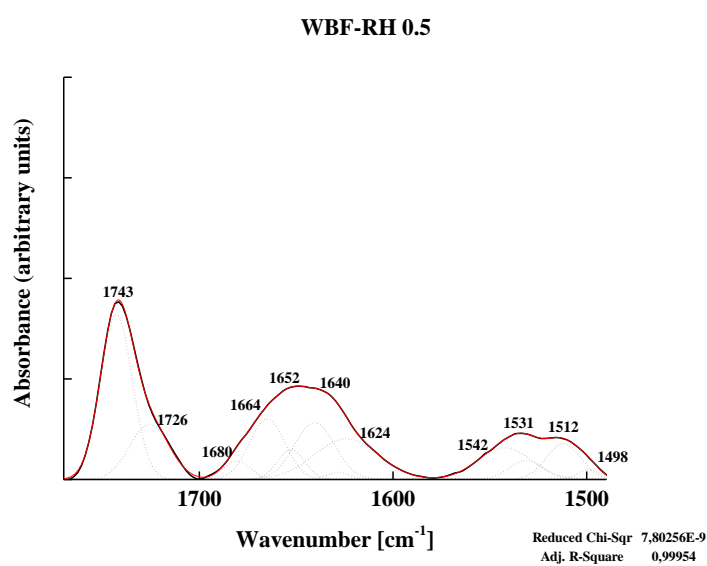
WBF-RH 0.5

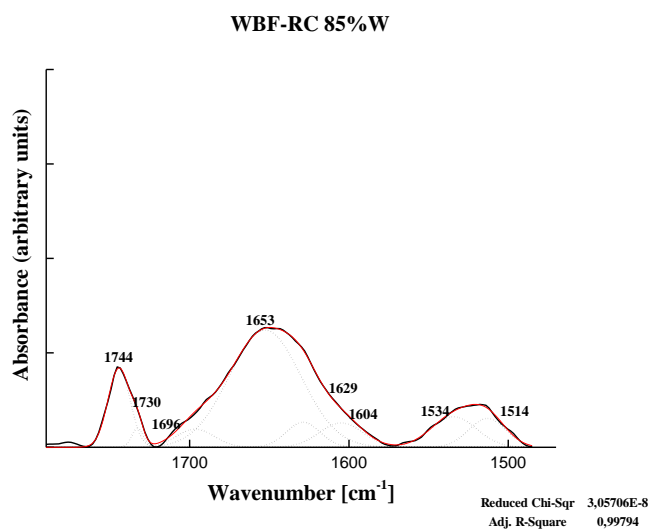*Figure 3.39 - FTIR spectra in the 1800–1450 cm^{-1} range for WBF-RH0.5 dough sample*

WBF-RH 1
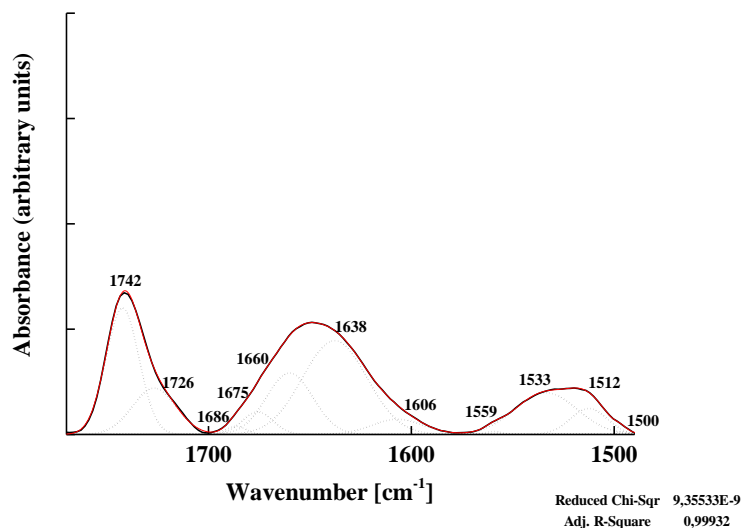
Figure 3.40 - FTIR spectra in the 1800–1450 cm⁻¹ range for WBF-RH1 dough sample

WBF-RH 1.5
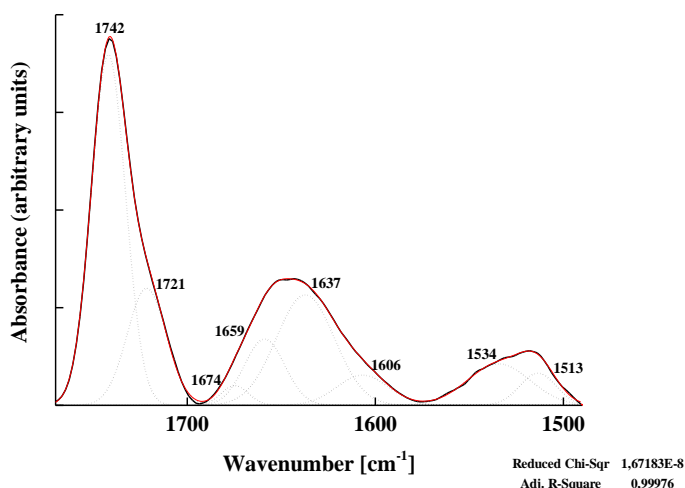
Figure 3.41 - FTIR spectra in the 1800–1450 cm⁻¹ range for WBF-RH1.5 dough sample

Effect of water addition on HPMC samples

The increase in water content did not alter the overall intensity of the amide I and amide II bands, but resulted in the appearance of additional sub-peaks around 1637-1638 cm⁻¹ (*β-sheet weakly bonded*), and 1609-1610 cm⁻¹ (NH_2^+ hydrated extended chains), which were not present in the sample at 70% water (Figure 3.40). Moreover, increasing the water content up to 100% (Figure 3.43) resulted in a more irregular shape of the amide II band and an increased signal of the band around 1741 cm⁻¹.
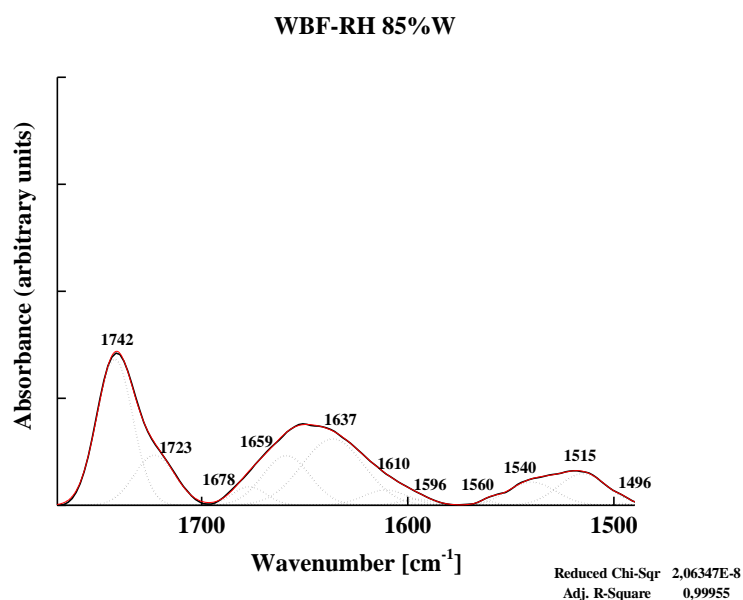
Figure 3.42 - FTIR spectra in the 1800–1450 cm⁻¹ range for WBF-RH85% dough sample
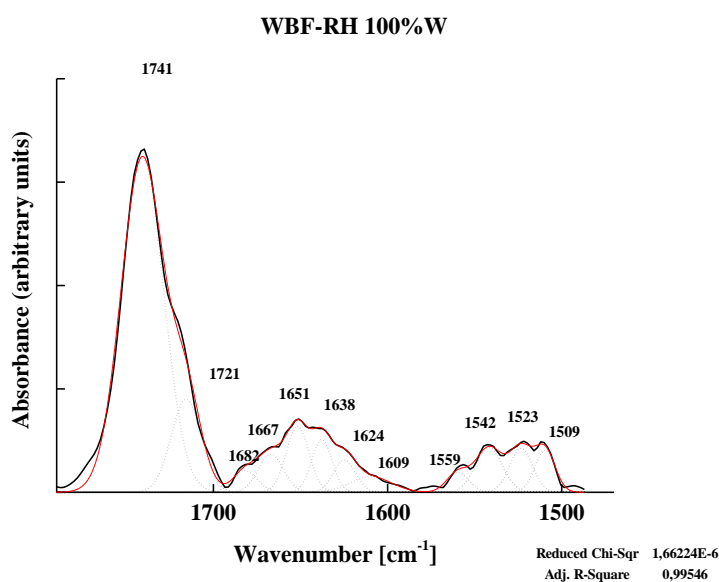
WBF-RH 100%W

Figure 3.43 - FTIR spectra in the 1800–1450 cm⁻¹ range for WBF-RH100% dough sample

Effect of CMC addition

In the samples prepared with CMC, the addition of the hydrocolloid seemed to cause a slight increase in the amide I and amide II band intensities. With respect to the changes observed in the materials, comparing the behaviour with the control WBF-R sample, the added formulations showed predominant β-sheet conformation (1631-1639-1635 cm⁻¹). At the highest CMC concentration, a predominant β-turn conformation is observed (1644 cm⁻¹) but, in this case, a gradual decrease and a final disappearance of the α-helix (1656 cm⁻¹) conformation also occurs.

WBF-RC 0.1

Figure labels on plot: 1742, 1719, 1674, 1656, 1631, 1597, 1558, 1542, 1515, 1507

Y-axis: **Absorbance (arbitrary units)**
X-axis: **Wavenumber [cm⁻¹]** with markers 1700, 1600, 1500

Reduced Chi-Sqr   9,41437E-8
Adj. R-Square   0,9982
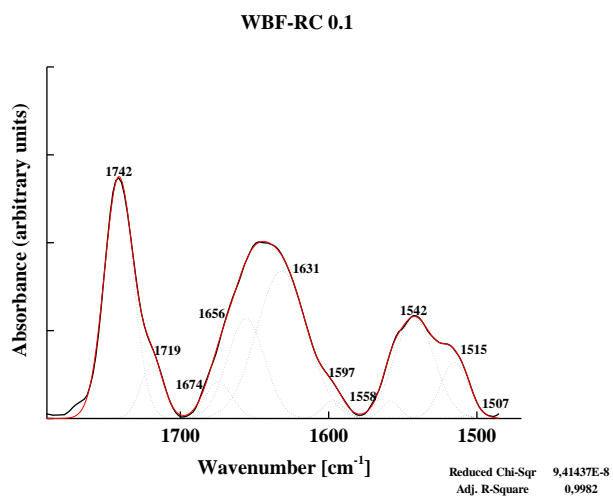
Figure 3.44 - FTIR spectra in the 1800–1450 cm⁻¹ range for WBF-RC 0.1 dough sample

WBF-RC 0.5

Figure labels on plot: 1742, 1719, 1662, 1680, 1639, 1604, 1538, 1528, 1514, 1498

Y-axis: **Absorbance (arbitrary units)**
X-axis: **Wavenumber [cm⁻¹]** with markers 1700, 1600, 1500

Reduced Chi-Sqr   8,37995E-8
Adj. R-Square   0,99905
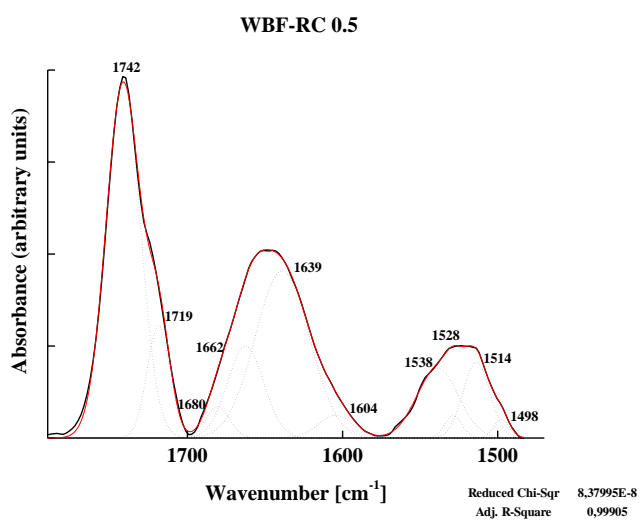
Figure 3.45 - FTIR spectra in the 1800–1450 cm⁻¹ range for WBF-RC 0.5 dough sample

WBF-RC 1

Figure labels on plot: 1742, 1722, 1684, 1663, 1635, 1600, 1551, 1528, 1514, 1500

Y-axis: **Absorbance (arbitrary units)**
X-axis: **Wavenumber [cm⁻¹]** with markers 1700, 1600, 1500

Reduced Chi-Sqr   2,63645E-8
Adj. R-Square   0,99931
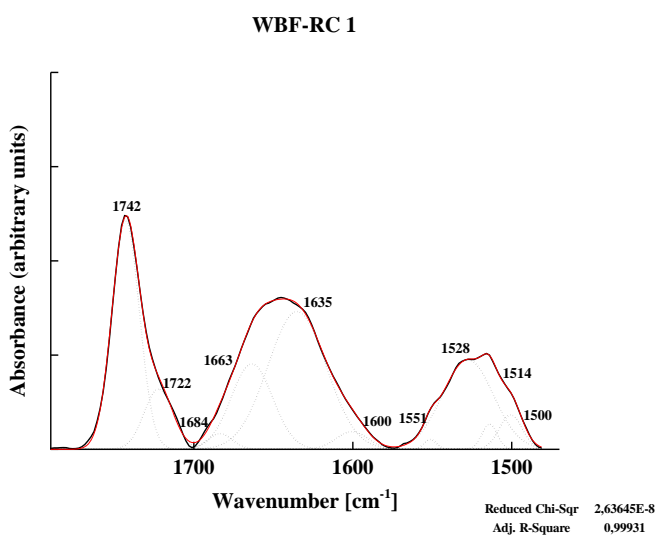
Figure 3.46 - FTIR spectra in the 1800–1450 cm⁻¹ range for WBF-RC 1 dough sample

WBF-RC 1.5

Reduced Chi-Sqr   8,25466E-8
Adj. R-Square   0,99649
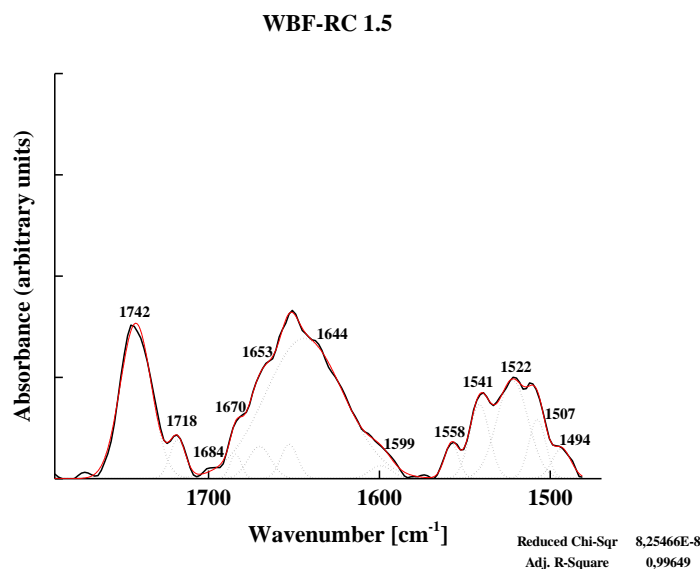
Figure 3.47 - FTIR spectra in the 1800–1450 cm^{-1} range for WBF-RC 1.5 dough sample

Effect of water addition on CMC sample

The addition of water reduced the overall amide I band signal, especially in the sample at 100% added water (Figure 3.49), when compared to the WBF-R1C sample (Figure 3.46). In the spectrum for *WBF-RC85%W* (Figure 3.48), a predominance of α-helix is seen (1653 cm^{-1}) while minor β-sheets peak (1629 cm^{-1}) and a peak at 1604 (NH$_2^+$ hydrated extended chains) are also observed.

In the WBF-RC100%W sample (Figure 3.49), the α-helix band has reduced intensity and is slightly shifted to 1659 cm^{-1}. Another sub-peak, assigned to weakly bonded β-sheets (1636 cm^{-1}) is also observed. Also for WBF-RC100%W, the peak at 1603 (NH$_2^+$ hydrated extended chains) is visible.

WBF-RC 85%W

Reduced Chi-Sqr   3,05706E-8
Adj. R-Square   0,99794

Figure 3.48 - FTIR spectra in the 1800–1450 cm^{-1} range for WBF-RC85%W dough sample

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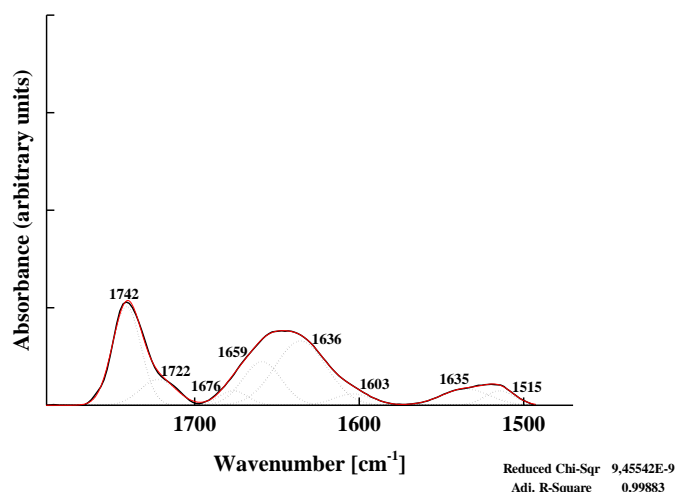
WBF-RC 100%W

Figure 3.49 - FTIR spectra in the 1800–1450 cm^{-1} range for WBF-RC 100%W dough sample

3.3.6 Porosimetry

The quantity of a gas or vapour adsorbed on a material is proportional to a surface area, and this could lead one to assume that the amount of adsorbed water could be related to the adsorbent specific area (i.e. to the fineness of the powder) but in dry foods, it is still unclear whether the mechanism involved is adsorption or absorption. It is rare to observe that the equilibrium amount of water taken up is dependent on the specific surface area of a dry food. Generally, the term 'sorption' is used, leaving the mechanism involved out of consideration (Walstra, 2003).

In this section, after analysing the raw materials, results related to bread crumbs will be discussed with the aim of understanding how the heat and composition could affect the baked materials in terms of specific surface area. Before obtaining consistent data in accordance with literature, there have been several instances of curves with irregular shapes, negative slopes of the linear section etc. within this study. The reasons for these results could be linked to various factors, such as the relatively high amount of the sample in the bulb (required for a non-porous material), the equilibrium time used, the reference sorbent usually used in the apparatus (silica alumina, which is more suitable for analysing high-porous materials). However, some of the apparent anomalous results obtained could be most correctly ascribed to the fact that the analysed materials are non-porous and with a very low surface area. To try to improve the results, set-up conditions, such as temperature, equilibration time and sample mass were modified several times, leading, at the end, to reasonable isotherms with Type II shapes, for most of the samples analysed.

As an example, Fig.3.50 represents the isotherm obtained for nitrogen adsorption and desorption on a commercial wholemeal flour (Allison Wholemeal Plain Flour, purchased from

Sainsbury's), which was used as a reference. Similar isotherms were obtained for all other raw materials and dried bread crumbs.

The shapes of these curves are characteristic of the widely known adsorption isotherm Type II, in accordance with the classification by Brunauer et al. (1938); such behaviour is most frequently observed when adsorption occurs on:

- non-porous powders or
- powders with pore diameters exceeding the dimensions of micropores.

The inflection point occurs near completion of the first adsorbed monolayer.
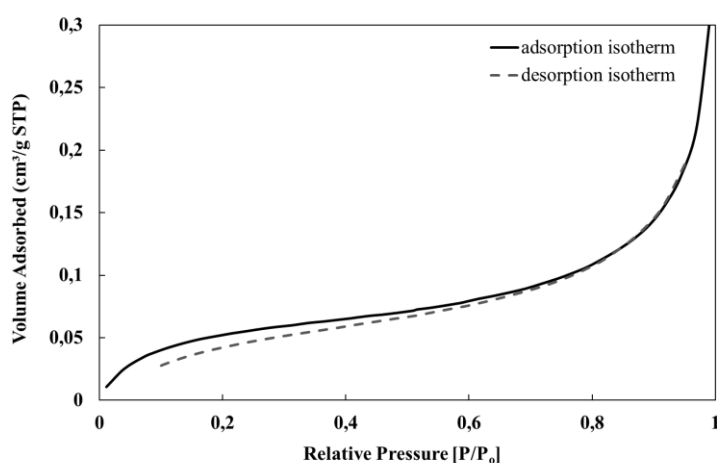
Figure 3.50 - Adsorption (solid line) and desorption (dashed line) isotherms of nitrogen for a whole wheat flour.

During the first phase of the adsorption process, at low relative pressure and at low adsorbate pressures, a monomolecular layer of absorbed substance is formed on the surface of the adsorbent. Volumetric pore filling (condensation in micropores) will also occur.

In this case, this part of the adsorption process is relatively short while the volume of adsorbed substance is very small. This leads to the conclusion that the main portion of the adsorbed material is used to form the monomolecular layer while volumetric pore filling plays a minor role or is quite non-existent. This assumption is borne out by the literature, which states that micropores are characteristic of adsorbents with considerable specific surface area sorption, and are closely linked with multilayer structure formation.

Fig.3.51 compares the adsorption isotherms obtained for some of the raw materials.
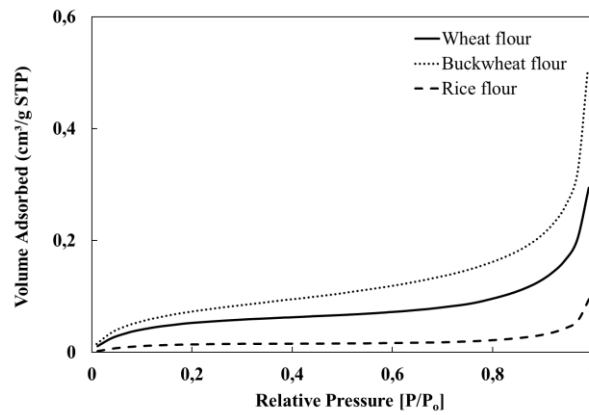
Figure 3.51 Adsorption isotherms of nitrogen for wheat flour (solid line), buckwheat flour (dotted line), rice flour (dashed line)

Results, in terms of BET surface areas, for the raw materials are shown in Figure 3.52
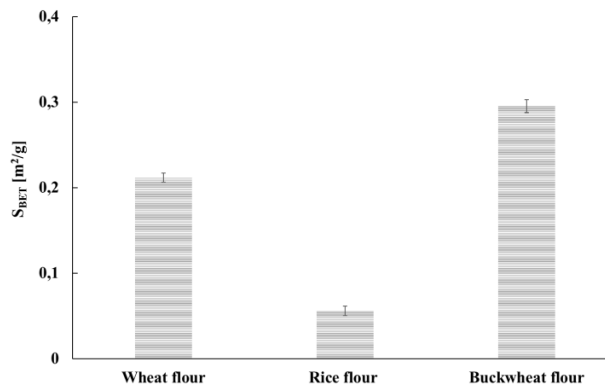
Figure 3.52 - S_{BET} of dry raw materials powders

Rice flour exhibited the lowest surface area compared to wheat and buckwheat flours. The large water uptake observed for some of the gluten free flours (e.g. buckwheat) could be related to their higher surface areas compared to wheat flour.

Results for the control breads crumbs are reported in Figure 3.53. WBF and R samples seem to have higher surface areas when compared to wheat flour. The WBF-R sample, resulting from the combination of the two gluten free flours, seems to exhibit a surface area similar to that for the glutenous reference.
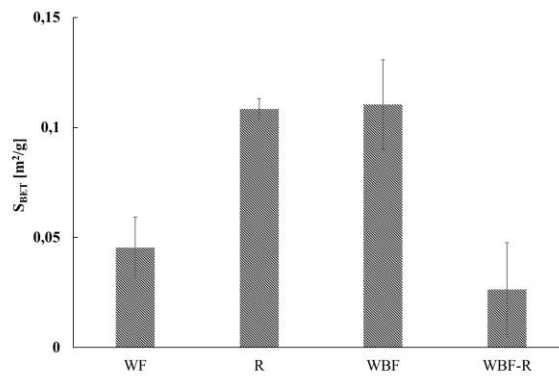
Figure 3.53 - S_{BET} of references samples (discussed in chapter 1)

Effect of hydrocolloid addition

Adding hydrocolloid to the WBF-R sample led to an increase in the specific surface area in both cases (CMC and HPMC addition). The similar trend observed for both the hydrocolloids could suggest that introducing them into the formulation has an effective and similar impact on the rearrangement of the bonds among the biopolymer chains within the material, which contributes to the formation of the final bread macro-structure.
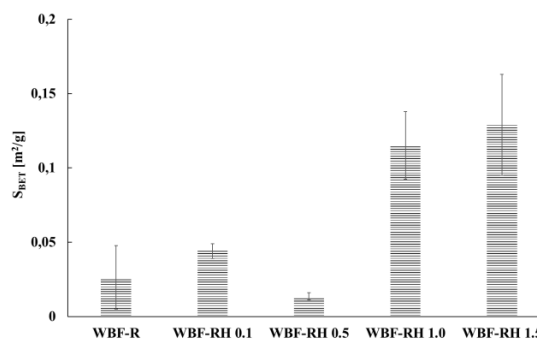
Figure 3.54 - S_{BET} for samples prepared adding HPMC at different level (Sample discussed in chapter 1)
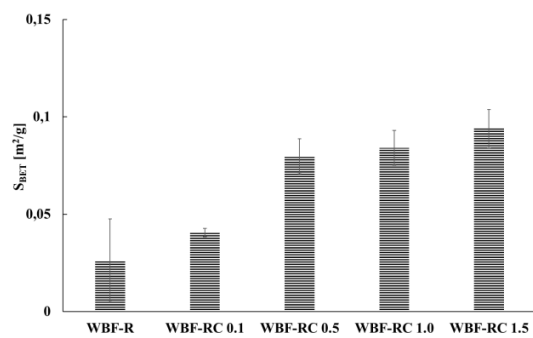
Figure 3.55 - S_BET for samples prepared adding CMC at different level (Samples discussed in chapter 1)

Effect of water addition

Increasing the amount of water up to 100% seems not to affect significantly the specific surface area for both the hydrocolloids but it affects the standard deviation. A lower standard deviation is observed in the WBF-RH100%W sample indicating that an increase in the water level, in the case of HPMC, led to a more homogeneous bread dough while in the case of CMC this can be observed in the sample at 85% of water.
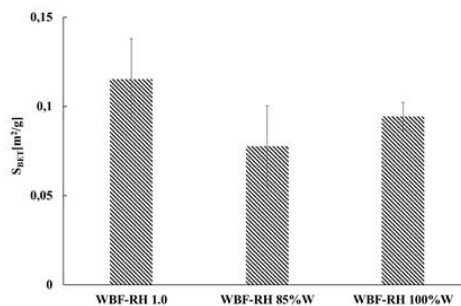
Figure 3.56 S_{BET} for samples at 1% HPMC and at different water concentration (Samples discussed in chapter 1)
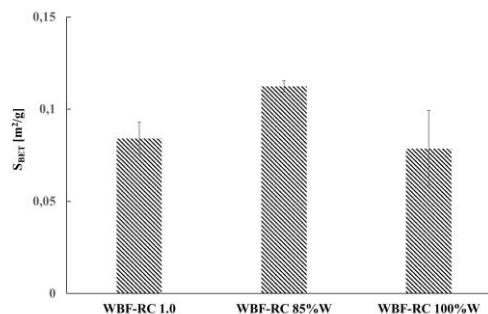
Figure 3.57 - S_{BET} for samples at 1% CMC and at different water concentration (Samples discussed in chapter 1)

3.3.7 SEM Analysis

The scanning electron microscopy image analysis method was used to obtain qualitative information on the characteristic surface morphology of some of the studied gluten-free samples. Results for CMC were similar to those for HPMC, although clearer images were obtained for samples prepared with HPMC. These are shown in Figure 3.59.

Microscopic observations of the microstructure of the control WBF-R sample, in the absence of hydrocolloid at higher magnification (Figure 3.58(c)), show a smoothed surface

when compared to the sample made with hydrocolloids. The addition of HPMC resulted in a more irregular matrix compared to WBF-R, probably due to the added cellulosic fibre component, which contributes to development of an improved protein-fibre-starch network with increasing water content. In this network, gelatinized starch granules are dispersed on the surface. In Figure 3.59(c)) some discontinuities are also observed as a consequence of the formation of a film-like structure, suggesting that the added hydrocolloids, at higher water contents, had a certain positive effect on stabilizing the gas cell during fermentation and baking.

WBF-R
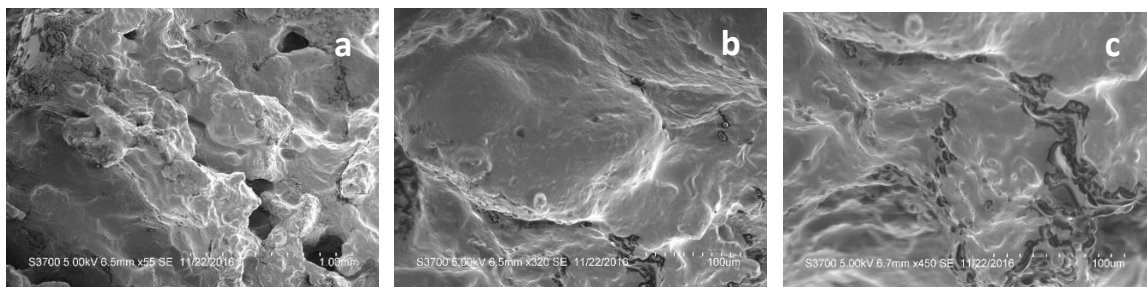
Figure 3.58 Scanning electron microscopy inside of the gluten-free bread crumb for WBF-R sample, under three different magnification a) 55X, b)320X, c)450X.

WBF-RH1   WBF-RH 85%W   WBF-RH 100%W
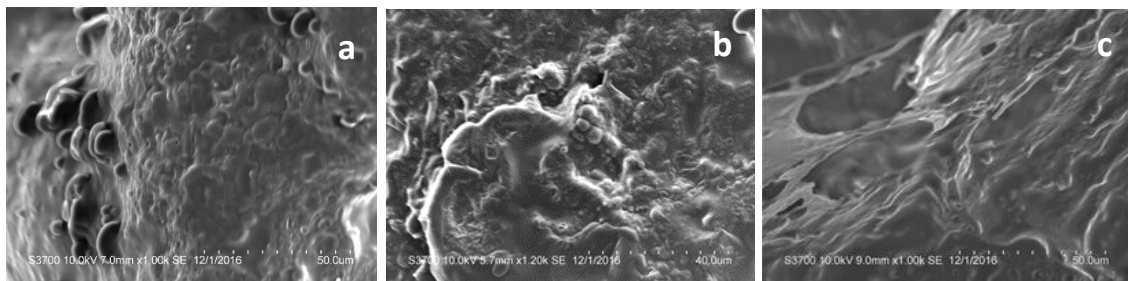
Figure 3.59 Scanning electron microscopy inside of the gluten-free bread crumb for a) WBF-RH1; b) WBF-RH 85%W; c) WBF-RH 100%W.

Conclusions

In this chapter the physico-chemical characteristics of GF dough, prepared using buckwheat and rice flours, and by adding hydrocolloids derived from cellulose (which also assumes the role of fibre), were studied by thermogravimetric analysis. The aim was to improve technological aspects of the GF breads, while also improving the nutritional quality, by increasing fibre content. This technique allowed determination of how the water distribution changes, modifying the composition of the system, can be effective in detecting the capacity of

the hydrocolloids to improve dough water retention by delaying early water loss, shifting it to higher temperatures.

A delay in water leakage may, somehow, be related to a delay in mass transfer, due to network formation, similar to those that form in glutenous doughs. These changes, in the delay of water loss, are ascribed to the formation of a protein-polysaccharides network within the biopolymers of the GF dough system, and this was also quantified in terms of activation energy by modelling the loss of water through semi-empirical non-isothermal drying kinetics equations.

Thermal analysis results showed that an increase in the hydrocolloid concentration generally increased the ability of the mixture to retain water, for both CMC and HPMC, with less uncertainty in the interpretation of data for the HPMC. An increase in water content in the samples obviously modifies the water loss rate, but no significant differences were observed in terms of water partitioning and peak temperatures.

FT-IR analysis, through the deconvolution of the amide I and amide II bands, showed changes in the conformations of the secondary protein structure. The addition of polysaccharides derived from cellulose led to changes in the amide I and II bands, and an increase in the concentration of hydrocolloids generally resulted in a reduction in α-helix conformation and in the appearance of β-sheets (strongly and weakly bonded). This suggests, in agreement with other theories, the possibility of the formation of a complex, due to a heated, induced interaction between the proteins and starches naturally contained in flour and the added polysaccharides, but this aspect need to be investigated further.

Porosimetry confirmed the effect, at the microstructural level of an increase in the concentration of hydrocolloids. Changes and improvements in the microstructure of the material, due to the addition of both hydrocolloids and water, which both, influenced the macroscopic characteristics of the final baked bread, were also confirmed by SEM analysis.

References

- Averill, B. A., Moulijn, J. A., van Santen, R. A., & van Leeuwen, P. W. N. M. (Eds.). (1999). *Catalysis: An Integrated Approach*. Elsevier. pp. 549

- Akoy, E. O. M. (2014). Experimental characterization and modeling of thin-layer drying of mango slices. *International Food Research Journal*, *21*(5).

- Bajwa, G. S., Sammon, C., Timmins, P., & Melia, C. D. (2009). Molecular and mechanical properties of hydroxypropyl methylcellulose solutions during the sol: gel transition. *Polymer*, *50*(19), 4571-4576.

- Barth, A. (2007) Infrared spectroscopy of proteins. *Biochimica Et Biophysica Acta-Bioenergetics*, 1767, 1073-1101

- Brunauer, S., P. Emmett & E. Teller (1938) Adsorption of gases in multimolecular layers. *Journal of the American Chemical Society*, 60, 309-319.

- Byler, D. M., & Susi, H. (1986). Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers,* 25(3), 469-487.

- Cai, J., & Chen, S. (2008). Determination of drying kinetics for biomass by thermogravimetric analysis under non-isothermal condition. *Drying Technology*, 26(12), 1464-1468.

- Carbonaro, M., & Nucara, A. (2010). Secondary structure of food proteins by Fourier transform spectroscopy in the mid-infrared region. *Amino acids*, 38(3), 679-690.

- Cerna, M., A. Barros, A. Nunes, S. Rocha, I. Delgadillo, J. Copikova & M. Coimbra (2003) Use of FT-IR spectroscopy as a tool for the analysis of polysaccharide food additives. Carbohydrate Polymers, 51, 383-389.

- Crockett, R., Ie, P., & Vodovotz, Y. (2011). How Do Xanthan and Hydroxypropyl Methylcellulose Individually Affect the Physicochemical Properties in a Model Gluten-Free Dough? *Journal of food science*, 76(3).

- Demirtas, C., Ayhan, T., & Kaygusuz, K. (1998). Drying behaviour of hazelnuts. *Journal of the Science of Food and Agriculture*, 76(4), 559-564.

- Dousseau, F., & Pezolet, M. (1990). Determination of the secondary structure content of proteins in aqueous solutions from their amide I and amide II infrared bands. Comparison between classical and partial least-squares methods. *Biochemistry*, 29(37), 8771-8779.

- Fessas, D., Schiraldi, A. (2001). Water properties in wheat flour dough I: classical thermogravimetry approach. *Food Chemistry*. 72, 237-244.143

- Fessas, D., Schiraldi, A. (2005). Water properties in wheat flour dough II: classical and Knudsen thermogravimetry approach. *Food Chemistry*. 90, 61-68.

- Fu, Y. C., Tong, C. H., & Lund, D. B. (2003). Moisture migration in solid food matrices. *Journal of food science*, 68(8), 2497-2503

- Guarda, A., Rosell, C. M., Benedito, C., & Galotto, M. J. (2004). Different hydrocolloids as bread improvers and antistaling agents. *Food hydrocolloids*, 18(2), 241-247.

- Guillen, M. D., & Cabo, N. (1997). Infrared spectroscopy in the study of edible oils and fats. *Journal of the Science of Food and Agriculture*, 75(1), 1-11.

- Hall, C. W. (2007). Reviews on drying: 1982–2006. *Drying Technology*, 25(1), 19-28.

- Hashimoto, A., & Kameoka, T. (2008). Applications of infrared spectroscopy to biochemical, food, and agricultural processes. *Applied Spectroscopy Reviews*, *43*(5), 416-451.

- Hayman, D. A., Sipes, K., Hoseney, R. C., & Faubion, J. M. (1998). Factors controlling gas cell failure in bread dough. *Cereal Chemistry*,

- Huang, H. C., Chen, L. C., Lin, S. B., & Chen, H. H. (2011). Nano-biomaterials application: In situ modification of bacterial cellulose structure by adding HPMC during fermentation. *Carbohydrate polymers*, 83(2), 979-987.

- Jekle, M., Mühlberger, K. & Becker, T. (2016) Starch–gluten interactions during gelatinization and its functionality in dough like model systems. *Food Hydrocolloids*, 54, Part A, 196-201.

- Kacurakova, M., P. Capek, V. Sasinkova, N. Wellner & A. Ebringerova (2000) FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses. Carbohydrate Polymers, 43, 195-203.

- Karoui, R., Downey, G., & Blecker, C. (2010). Mid-infrared spectroscopy coupled with chemometrics: A tool for the analysis of intact food systems and the exploration of their molecular structure− Quality relationships− A review. *Chemical reviews*, 110(10), 6144-6168.

- Kong, J. & S. Yu (2007) Fourier transform infrared spectroscopic analysis of protein secondary structures. Acta Biochimica Et Biophysica Sinica, 39, 549-559.

- Langmuir, I. (1916) The constitution and fundamental properties of solids and liquids Part I Solids. *Journal of the American Chemical Society*, 38, 2221-2295.

- Li, Z., & Kobayashi, N. (2005). Determination of moisture diffusivity by thermo-gravimetric analysis under non-isothermal condition. *Drying technology*, *23*(6), 1331-1342.

- Libnau, F. O., Kvalheim, O. M., Christy, A. A., & Toft, J. (1994). Spectra of water in the near-and mid-infrared region. *Vibrational spectroscopy*, *7*(3), 243-254.

- Lin, J., & Brown, C. W. (1992). Near-IR spectroscopic determination of NaCl in aqueous solution. *Applied Spectroscopy*, *46*(12), 1809-1815.

- Liu, X., Yu, L., Xie, F., Li, M., Chen, L., & Li, X. (2010). Kinetics and mechanism of thermal decomposition of cornstarches with different amylose/amylopectin ratios. *Starch-Stärke*, 62(3-4), 139-146.

- Lodi, A., & Vodovotz, Y. (2008). Physical properties and water state changes during storage in soy bread with and without almond. *Food Chemistry*. 110, 554-561.

- Matveev, Y. I., Grinberg, V. Y., & Tolstoguzov, V. B. (2000). The plasticizing effect of water on proteins, polysaccharides and their mixtures. Glassy state of biopolymers, food and seeds. *Food Hydrocolloids*, *14*(5), 425-437.*75*(5), 585-589.

- Mejia, C., L. Mauer & B. Hamaker (2007) Similarities and differences in secondary structure of viscoelastic polymers of maize alpha-zein and wheat gluten proteins. *Journal of Cereal Science*, 45, 353-359.

- Michell, A. J., & Schimleck, L. R. (1996). NIR spectroscopy of woods from Eucalyptus globulus. *Appita journal*, *49*(1), 23-26.

- Moreira, R., Chenlo, F., & Arufe, S. (2015). Starch transitions of different gluten free flour doughs determined by dynamic thermal mechanical analysis and differential scanning calorimetry. *Carbohydrate polymers*, 127, 160-167.

- Ndapeu, D., Njeugna, E., Bistac, S. B., Drean, J. Y., Fogue, M., & Foba, J. N. (2013). Experimental Study of the Drying Kinetics of the Coconut Shells (Nucifera) of Cameroon. *Materials Sciences and Applications*, *4*(12), 822.

- Nevskaya, N. A., & Chirgadze, Y. N. (1976). Infrared spectra and resonance interactions of amide-I and II vibrations of α-helix. *Biopolymers*, *15*(4), 637-648.

- Nielsen, S. S. (Ed.). (2010). *Food analysis* (pp. 530-535). New York: Springer.

- Özdemir, M., & Devres, Y. O. (1999). The thin layer drying characteristics of hazelnuts during roasting. *Journal of Food Engineering*, *42*(4), 225-233.

- Patino, J. M. R., & Pilosof, A. M. (2011). Protein–polysaccharide interactions at fluid interfaces. *Food Hydrocolloids*, 25(8), 1925-1937.

- Pushpamalar, V., Langford, S. J., Ahmad, M., & Lim, Y. Y. (2006). Optimization of reaction conditions for preparing carboxymethyl cellulose from sago waste. *Carbohydrate Polymers*, 64(2), 312-318.

- Ripoche, A., & Guillard, A. S. (2001). Determination of fatty acid composition of pork fat by Fourier transform infrared spectroscopy. *Meat Science*, *58*(3), 299-304.

- Roberts, J. S., & Tong, C. H. (2003). Drying kinetics of hygroscopic porous materials under isothermal conditions and the use of a first-order reaction kinetic model for predicting drying. *International Journal of Food Properties*, *6*(3), 355-367.

- Robertson, G., K. Gregorski & T. Cao (2006) Changes in secondary protein structures during mixing development of high absorption (90%) flour and water mixtures. *Cereal Chemistry*, 83, 136-142.

- Rojas, J. A., Rosell, C. M., & De Barber, C. B. (1999). Pasting properties of different wheat flour-hydrocolloid systems. *Food hydrocolloids*, *13*(1), 27-33.

- Sammon, C., Bajwa, G., Timmins, P., & Melia, C. D. (2006). The application of attenuated total reflectance Fourier transform infrared spectroscopy to monitor the concentration and state of water in solutions of a thermally responsive cellulose ether during gelation. *Polymer*, 47(2), 577-584.

- Sammon, C., Bajwa, G., Timmins, P., & Melia, C. D. (2006). The application of attenuated total reflectance Fourier transform infrared spectroscopy to monitor the concentration and state of water in solutions of a thermally responsive cellulose ether during gelation. *Polymer*, *47*(2), 577-584.

- Sarkar, N. (1979). Thermal gelation properties of methyl and hydroxypropyl methylcellulose. *Journal of applied polymer science*, 24(4), 1073-1087.

- Secundo, F., & Guerrieri, N. (2005). ATR-FT/IR study on the interactions between gliadins and dextrin and their effects on protein secondary structure. *Journal of agricultural and food chemistry*, 53(5), 1757-1764.

- Sinelli, N., E. Casiraghi & G. Downey (2008) Studies on proofing of yeasted bread dough using near- and mid-infrared spectroscopy. Journal of Agricultural and Food Chemistry, 56, 922-931.

- Sing, K. S. (1985). Reporting physisorption data for gas/solid systems with special reference to the determination of surface area and porosity (Recommendations 1984). *Pure and Applied Chemistry*, 57(4), 603-619.

- Sivam, A. S., Sun-Waterhouse, D., Perera, C. O., & Waterhouse, G. I. N. (2012). Exploring the interactions between blackcurrant polyphenols, pectin and wheat biopolymers in model breads; a FTIR and HPLC investigation. *Food Chemistry*, 131(3), 802-810.

- Sivam, A., D. Sun-Waterhouse, C. Perera & G. Waterhouse (2013) Application of FT-IR and Raman spectroscopy for the study of biopolymers in breads fortified with fibre and polyphenols. *Food Research International*, 50, 574-585.

- Taheri-Garavand, A., Rafiee, S., & Keyhani, A. (2011). Study on effective moisture diffusivity, activation energy and mathematical modeling of thin layer drying kinetics of bell pepper. *Australian Journal of Crop Science*, 5(2), 128.

- Thommes, M., K. Kaneko, A. Neimark, J. Olivier, F. Rodriguez-Reinoso, J. Rouquerol & K. Sing (2015) Physisorption of gases, with special reference to the evaluation of surface area and pore size distribution (IUPAC Technical Report). *Pure and Applied Chemistry*, 87, 1051-1069

- Tolstoguzov, V. (1997). Thermodynamic aspects of dough formation and functionality. Food Hydrocolloids, 11(2), 181-193.

- Tori, H. & M. Tasumi (1992) Model-alculation On The Amide-I Ifrared Bands Of Globular-Proteins. *Journal of Chemical Physics*, 96, 3379-3387.

- van Velzen, E., J. van Duynhoven, P. Pudney, P. Weegels & J. van der Maas (2003) Factors associated with dough stickiness as sensed by attenuated total reflectance infrared spectroscopy. *Cereal Chemistry*, 80, 378-382.

- Walstra, P. & H. Lyklema (2002) About Professor Gert Frens. Colloids and Surfaces a-Physicochemical and Engineering Aspects, 210, 127-128.

- Walstra, P. (2002). About Professor Gert Frens. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 210(2), 127-128.

- Yilbas, B., Hussain, M., & Dincer, I. (2003). Heat and moisture diffusion in slab products due to convective boundary condition. *Heat and Mass Transfer*, *39*(5), 471-476.

- Zheng, G. H., Sosulski, F. W., & Tyler, R. T. (1997). Wet-milling, composition and functional properties of starch and protein isolated from buckwheat groats. *Food research international*, *30*(7), 493-502.

<div align="right">Chapter 4</div>

Study of GF bread model systems based on resistant starch and vegetable proteins and treated with mTG-ase: Part I

Abstract

Gluten-Free (GF) products are mainly based on starch of various botanical origins, bringing a limited amount of proteins and fibres in the diet. Resistant starch (RS) represents an interesting alternative to the most commonly used starches due to its characteristics. It is classified as a dietary fibre, because it is not digested in the small intestine, and it can also help in preventing some serious diseases. In fact, many studies on humans have found that RS can have powerful health benefits: like insulin sensitivity, lower blood sugar levels, reduced appetite and various benefits for digestion, several beneficial effects on the colon, reduces the pH level, potently reduces inflammation and leads to several beneficial changes that should lower the risk of colorectal cancer etc. For this reason, it is considered as "Superfood". Moreover, due to the general low proteins quantity in the GF products, proteins from different botanical sources such as, soy and hemp, can be used in the enrichment of GF bread formulations with the role of nutrients and as structure and texture forming agents. However, their low ability to cross-link make them generally not considerable for baked goods. Then, to improve doughs nutritional value, texture and appearance, in the current work a study of GF bread formulations based on resistant starch, vegetable proteins and using mTG-ase, has been carried out.

The study investigates the possibility of obtaining a GF bread enriched in fibres and the impact of three proteins, used at different levels and treated with mTG-ase, on the bread macroscopic final structure by rheological investigation.
A whole-wheat flour bread recipe was used as a control. Wheat flour has been replaced with a mixture of RS and proteins added at different level (6%, 9% and 12%) as model doughs, and an investigation of the mechanical properties was performed studying oscillatory measurements

at different temperatures and employing a thermal ramp to obtain baking information, and by lubricated squeezing flow with the aim to obtain the extensional viscosity.

4.1 Introduction

The starch commonly found in bread is known to be rapidly digested and absorbed in the small intestine, producing undesirably high blood-glucose and high insulin levels after a meal (Jenkins et al., 1981). This problem becomes even more serious in patients who must follow a GF diet, because, as already widely discussed in Chapter 1, the GF baked products are nutritionally unbalanced, with a high starch content. Moreover, these products lack in fibres and other nutrients.

Nowadays, the importance given to high quality food products obtained with functional ingredients, is growing. These ingredients are responsible of reducing the risk of some diseases like the level of cholesterol, inflammation of the intestine, etc. Resistant starch (RS) can also be considered a functional ingredient. It is becoming popular in the production of GF baked goods due its documented positive effects on the digestive system, the microbial flora, the blood cholesterol level and the control of diabetes (Nugent 2005, Higgins, 2004).

RS is intrinsically indigestible, so it acts like a soluble fibre even if traditional dietary fibre are non-starch polysaccharides (Trowell, 1976). The fibre-like behaviour of RS is due to its compact structure given by the high amylose content, which limits the accessibility of the digestive enzymes (Sajilata et al., 2006). While in a normal starch the amylose content is about 20-25% (Björck et al., 1990) in the resistant starch it can reach about 60% or more. Including this type of starch in food formulations, it has been observed a positive effetc on sensory and technological properties of final baked goods, providing them with a better appearance, texture, and mouthfeel than conventional fibers (Fuentes-Zaragoza et al., 2010). Quality improvements have been obtained using RS in the production of both gluten and GF products as reported in literature (Fuentes-Zaragoza et al., 2010, Korus et al., 2009; Ozturk et al., 2009; Brown 2004).

Vegetable proteins are also widely used in the enrichment of GF bread formulations with the role of nutrients and as structure and texture forming agents, in substitution of animal proteins.

Several studies have indeed pointed out the beneficial effect of a vegetable protein based diet, in comparison to an animal protein diet, in preventing and ameliorating some diseases (Kristensen et al., 2016; Bianchi et al., 1993; Breslau et al., 1988) Proteins also affects positively structure, texture, sensory properties as well as nutritional quality, and reduce the amino acid deficit (Gerard et al., 2002; Marshall, 2004).

The combination of structuring and gelling properties of both resistant starch and vegetable proteins to obtain GF foods would represent a good starting point to formulate a technologically advanced, healthier, and nutritionally balanced product.

New trends in the food field are moving in this direction even though from a scientific point of view very little exists in literature.

Soy is only recently becoming more common in food industry. From the last studies, it emerged that most of the world's soybeans are processed or crushed into soybean meal and oil (Ali 2010). It is estimated that only the 2% of soybean production is consumed by humans, directly as food (Hartman et al., 2011), the rest is used for animal feed. Soy proteins can form gels with good water holding ability upon heating, (Chen et al., 2017; Renkema et al., 2000). Soy flour has long been recognized as an excellent additive to fortify cereal products (bread, cookies, crackers and pasta).

Its significant levels of high-quality proteins, rich in essential amino acids, provides health benefits effect (reduce risk of coronary heart disease, osteoporosis, menopausal and postmenopausal women symptoms, and helps in cancer prevention) (Costantini et al. 2014).

Hemp's storage proteins consist mainly of edestin (globulin) and albumin, with a high quality essential amino acid profile and high digestibility (Tang et al., 2006). Defatted hemp flour was used in the past for the fortification of energy bars (Norajit et al., 2011). The high content of methionine and cystine residues in hemp increases the extent of protein aggregation thanks to the formation of covalent disulphide bonds between individual molecules (Tang et al., 2006).

Various enzymes such as amylases or proteases are known to create positive effects on the quality of baked products (Gupta et al., 2003; Keskin et al., 2004), while microbial transglutaminase (m-TGase) is a relatively new tool used in the manufacture of baked goods. mTG-ase is a simple monomeric protein and its activation requires no special cofactors. It has an optimum pH 5-8 and temperature 50-55°C and loses activity at 70°C (Yokoyama et al., 2004). Generally, m-TGase is more used in meat, fish and dairy industry (Motoki and Seguro, 1998; Kuraishi et al., 1998; Jiang et al., 1998). Transglutaminase has a unique ability to modify proteins functionality, promoting proteins cross-linking, catalysing the formation of covalent bonds between the glutamyl and lysine residues in proteins (Yokoyama et al., 2004). The mTG-ase reacts differently with various protein sources and at different addition levels (Moore et al., 2006). Cross-links interactions promoted by mTG-ase, causes the formation of large protein aggregates, and this is associated with lowering extensibility and increasing resistance of doughs (Huang et al., 2008). Good substrates for mTG-ase are those with glutamine in flexible regions of polypeptide chain (casein) or in the region of reverse turns (soy, pea, lupin, rice and

sunflower) (Dube, et al., 2007). In contrast, globular proteins such as ovalbumin and β-lactalbumin, which in their native state are stabilized by disulphide bonds, cannot be attacked by m-TGase.

Very little is reported on the effects of m-TGase on the GF breads structuration using proteins from vegetable sources. Then, on the light of the above, aim of the present study is to examine the possibility to obtain baked goods by using vegetable proteins and resistant starches, eventually adding the m-TGase to improve the network inside the dough.

The ability to cross-link of two vegetable proteins were studied in this research performing a rheological characterization on different reference samples made with soy and hemp proteins and RS at different percentage and adding in a second step m-TGase. In fact, an induced aggregation is expected by the m-TGase due to their substrate.

4.2 Materials and methods

4.2.1 Bread ingredients and Samples preparation

The resistant starch (RS) used in this work was an Hi-Maize 260 with 56% of amylose and was kindle supplied by Ingredion (UK Limited); hemp protein meal (HP) (47% of hemp protein) and soy protein meal (SP) (91% of soy protein) were purchased by BulkPowered (Unit 1 Gunfleet Business Park, Colchester, CO4 9QX) and a food enzymatic preparation (HI-NET SUPREME Q) based on pure Transglutaminase (m-TGase) were kindle supplied by HI-Food (Parma, Italy); the other ingredients were: salt (Italkali, Italia), sunflower oil , tap water and fresh yeast (Conad, Italia).

All samples were prepared using the same procedure. Dry ingredients (starch powder, salt, protein meal, and m-TGase when added) and fats were mixed together for 1 min using a N50 Hobart mixer (Hobart Corporation, USA), water was preheated at 40°C and then added to the premixed ingredients. After adding water, the dough was mixed for further 15 min. No yeast was added to prepare dough samples for rheological test, to prevent bubbles formation which could interfere with the measurements. Fresh yeast (3.3 % w/w) was used to prepare doughs for baking tests, dissolving it in the preheated water and then adding to the mixture of solids.

Samples prepared for baking test were proofed at 40°C and baked for 45 min at 185 °C. All studied samples are resumed in Table 4.1-4.2. The m-TGase was tested at only one concentration and was added at 0.25% (on protein content basis). As it is possible observe, the soy and hemp protein quantity was increased from 6% to 12%.

	HP6%	HP9%	HP12%	SP6%	SP9%	SP12%
RS	314	291	268	336	324	312
HP	46	69	92	-	-	-
SP	-	-	-	24	36	48
salt	2	2	2	2	2	2
W	360	360	360	360	360	360
O	35	35	35	35	35	35

Table 4.1 Control samples

	HP6%TG	HP9%TG	HP12%TG	SP6%TG	SP9%TG	SP12%TG
RS	314	291	268	336	324	312
HP	46	69	92	-	-	-
SP	-	-	-	24	36	48
TG	0.054	0.081	0.108	0.054	0.081	0.108
salt	2	2	2	2	2	2
W	360	360	360	360	360	360
O	35	35	35	35	35	35

Table 4.2 – Treated samples

Protein-Water solution

Simple proteins solutions at different protein concentration have also been studied to understand the phenomena related to the only protein. The samples were obtained by mixing water (W) and protein meal (HP and SP) for 15 min. Studied solutions are resumed in Table 4.3

	HP6% Sol	HP9% Sol	HP12% Sol	SP6% Sol	SP9% Sol	SP12% Sol
HP	6	9	12	-	-	-
SP	-	-	-	6	9	12
W	94	91	88	94	91	88

Table 4.3 Water-Protein solutions

4.2.2 Rheological characterization ad fitting model

Protein-Water solutions analysis

Time cure tests were performed on protein-water solutions, using a controlled strain rheometer ARES-RFS (TA Instrument, USA), in linear condition and at 1 Hz, increasing temperature from 20°C to 90°C for soy solution and 60°C for Hemp solution, with a ramp rate of 1 ° C/min. The functionality of the complex modulus G* with the protein concentration and temperature was obtained with the aim to understand the temperature transition.

GF Doughs analysis

The rheological characterization on dough samples was performed by using a controlled strain rheometer ARES-RFS (TA Instrument, USA). The temperature control is guaranteed by a Peltier system, acting under the lower plate. The rheometer is equipped with a parallel plate geometry (Φ = 25mm) using a gap of 2.1±0.1mm. The gap used during the tests was approximately 2.0±0.2 mm. Frequency sweep tests were performed increasing frequency from 0.1 Hz to 10 Hz, in the linear viscoelastic region, previously determined by time sweep and stress sweep tests. The tests were performed for all the samples at three temperatures: 25°C, 50°C, and 70°C. Time cure tests in linear regime were also run at 1 Hz, increasing temperature from 20°C to 95°C, with a ramp rate of 1 °C/min. When running tests at high temperature, water loss was prevented covering the sample rim with a thin layer of silicon oil (viscosity 1 Pa·s, VWR Chemicals, France).

Each sample was prepared twice, and each test was carried out in triplicate, all data are shown in terms of mean value and standard deviation.

From a rheological point of view, dough is a very complex system, often described as a solid-like material that behaves as a "weak gel" in the frequency range usually investigated (0.1-100 Hz) (Gabriele et al, 2001; Migliori & Gabriele, 2010; Ng & McKinley, 2008; Peressini et al, 2002). According to the "weak gel model", the material can be described, in a schematic way, as a three-dimensional network formed by interacting rheological units. As a consequence of this model, the dynamic moduli vs frequency trend in a double log plot is linear and the complex modulus, i.e. the combination of G' and G", can be fitted by a power law (Gabriele et al, 2001):

$$G^*(\omega) = \sqrt{(G')^2 + (G'')^2} = A \cdot \omega^{\frac{1}{z}} \quad (4.1)$$

where A is related to the strength of the three-dimensional network and z is a measure of the network extension.

4.2.3 Determination of the onset of gelatinisation temperature (T_o)

With the aim of determining the critical temperatures, corresponding to the gelatinization phenomena, the G' trend was analyzed in detail. The G' transition is related to the solid-like behavior and, therefore, it seems more linked to the effect of the structure development. According to a procedure already proposed in the literature for different starch based systems (Marino et al, 2014) for both the type of samples (protein-water solutions and GF doughs), the onset of gelatinisation temperature (T_o) was obtained from of the knee point of G' curve considering the tangent to the curve inflection point as shown in Figure 4.1.
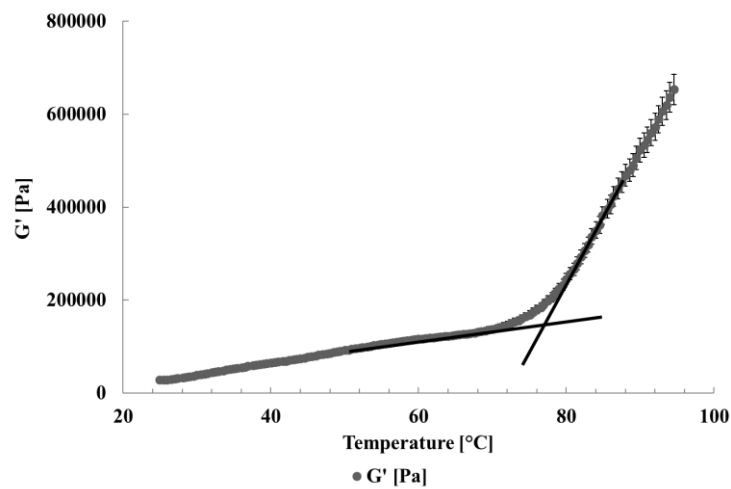
Figure 4.1 G' modulus vs temperature for RS sample

4.2.4 Lubricated Squeeze flow

All experimental runs were performed according to the procedures described in Chapter 2 (section 2.2.5).

4.2.5 Baking tests and physical characteristics

For samples used in baking tests, at the end of the mixing process, the dough was placed into a baking tin, covered with foil and placed in a proofer at 40°C for 2 h. After proofing, loaves were baked in a fan oven (LineMiss XFT113, Unox, Italy) for 40 min at 180°C. The oven was preheated at 180°C for 5 min. All baked products were cooled down and analysed after they have reached the room temperature.

Colour appearance is a key quality indicator for many baked goods, therefore colorimetric measurements were carried out on crust after baking using a Minolta Colorimeter (Chroma-Meter CR-400, Konica Minolta Sensing, Osaka, Japan) using the CIELab colour system; obtained parameters are L* (lightness), a* (-green, + red), b* (-blue, +yellow) (Costantini et al, 2014). They were obtained as average of four different determinations in different points of sample (either crust or crumb).

4.2.6 Statistical analysis

All data are shown in terms of mean and standard deviation. A statistical analysis, when necessary, was carried out using one-way ANOVA and t-student test (MS Excel 2013, Microsoft, USA) and differences among compared samples were considered significant at p-value <0.05 (interval of confidence of 95%).

4.3. Results and discussion

4.3.1 Rheological properties of water-proteins solutions

Food systems have a multicomponent nature. Two types of macromolecular components, proteins and polysaccharides, are mainly responsible for the food structure. These macromolecules have important food-gelling and thickening ability, and, if put in biphasic systems, like emulsions or foams, they can act as stabilizers.
Moreover, proteins and polysaccharides can be co-soluble, incompatible or complexed in the same medium (De Kruif and Tuinier. 2001). Biopolymer incompatibility increases in the conditions favourable for self-association and enlargement of macromolecules (Tolstoguzov, 1991).

A preliminary study of simple water/protein solutions could be therefore useful in order to gain information about their rheological properties because the observable rheological transitions could help in understanding when and in which conditions (temperature and concentration) the self-association of the considered proteins occurs.

The results of the temperature ramp tests for the protein-water solutions at different protein concentrations are shown below.

From the temperature ramp test, the sample at the lowest soy protein concentration (SP6%Sol) showed a more liquid-like behaviour (Figure 4.2), whereas at higher concentrations (SP9%Sol and SP12%Sol) the behaviour is more solid-like (Figure 4.3). Transitions in the solutions are observed around 40°C in the SP6%Sol and at higher temperatures for solutions with a higher protein concentration.

For sample SP9%Sol the dynamic complex modulus and the phase angle are almost constant up to 70°C (Figure 4.3), after this temperature changes in the molecular mobility occur while for the samples SP12%Sol transitions seems to start at slightly lower temperatures.
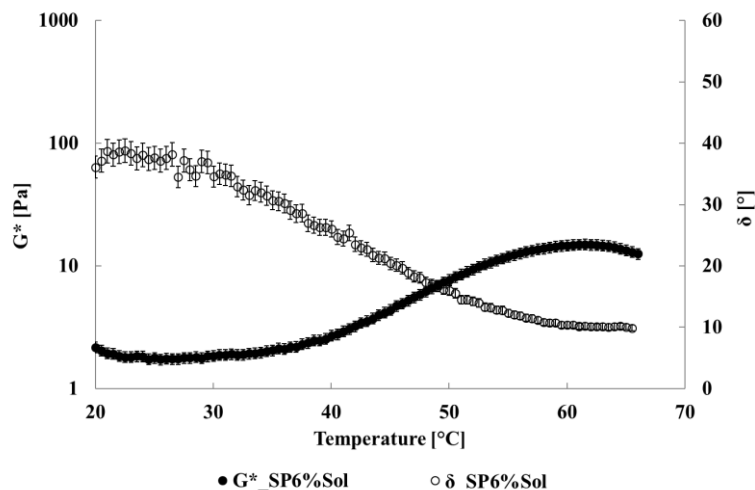
Figure 4.2 Complex modulus and phase angle vs temperature for SP6%Sol
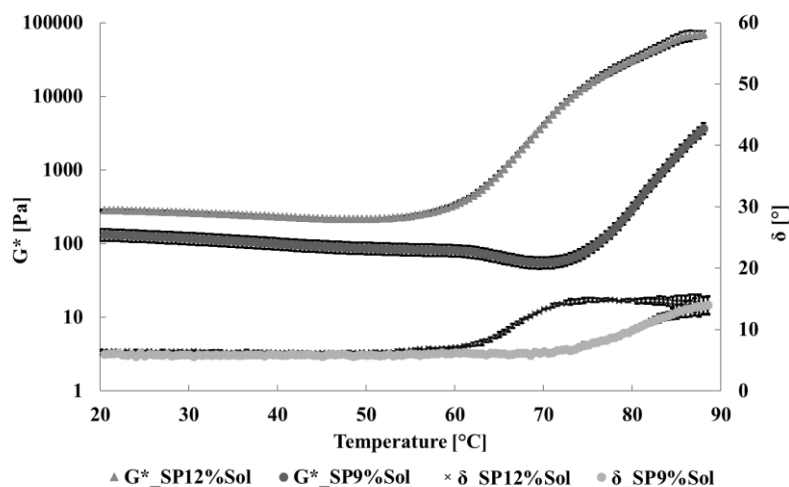
Figure 4.3 Complex modulus and phase angle vs temperature for SP9%Sol and SP12%Sol

For HP6%Sol and HP9%Sol, the G * and δ values remain constant up to 40 °C (Figure 4.4), whereas above 40 °C, for the HP9%Sol sample there is an increase in the G* and a consequential decrease in the phase angle, indicative of a more structured material. Also for the HP6%Sol sample between 40 and 45° C the same behaviour is observed. At higher temperatures, δ collapses, indicating a structuration of the material (Figure 4.4).
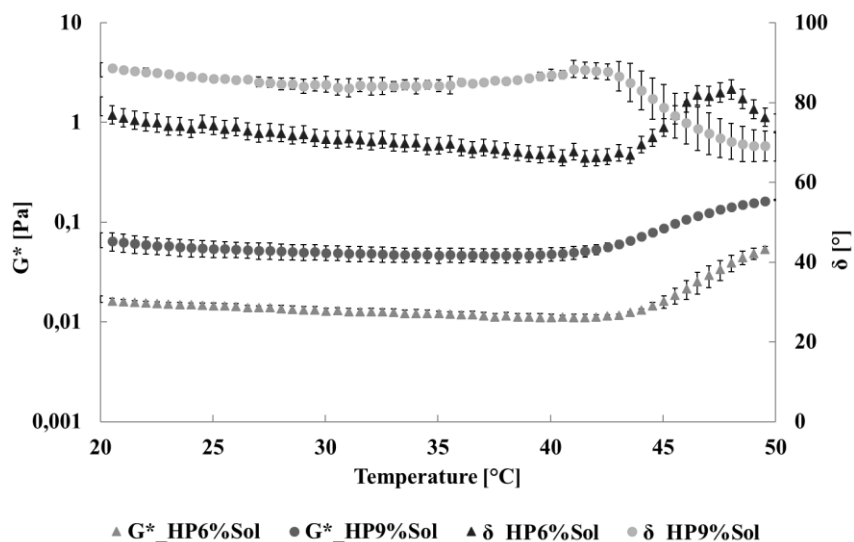
Figure 4.4 Complex modulus and phase angle vs temperature for different hemp solutions
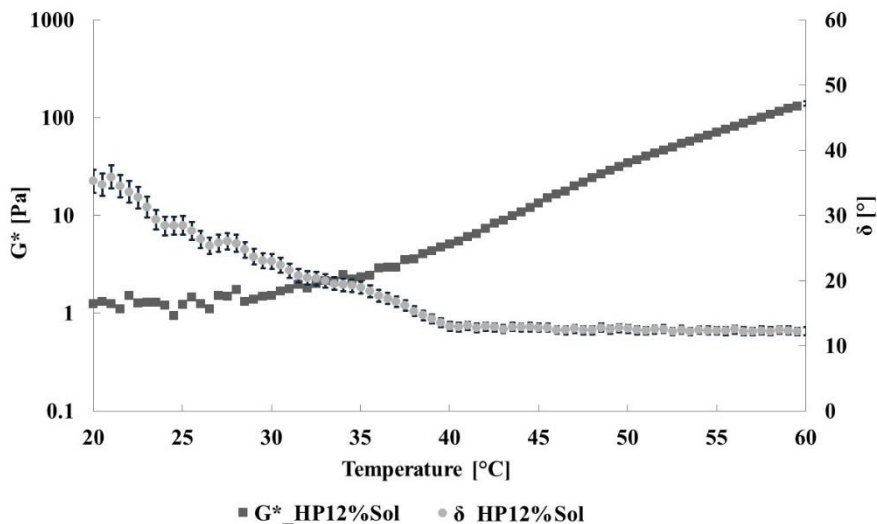
Figure 4.5 Complex modulus and phase angle vs temperature 12%HPsol

For the sample 12%HPSol the value of G* is constant up to 30° C; above this critical temperature value, G* increases with temperature, while the phase angle slight decreases. (Figure 4.5).

The beginning of the transitions, was estimated in terms of onset gelatinization temperature and results are resumed in table

Samples	T$_0$ [°C]
HP6%Sol	45.3±1,2
HP9%Sol	42.7±0.3
HP12%Sol	51.5±0.5
SP6%Sol	42,8±0.8
SP9%Sol	83.3±2.5
SP12%Sol	72.3±0.3

Table 4.4 T$_o$ for protein-water solutions at different protein concentration

From table 4.4 for both soy and hemp an increase in protein concentration lead to an overall increase in the onset gelatinization temperature. At low concentration (6% of protein) the protein self-aggregation in the solution seems to occur at the same temperature for both soy and hemp. In the case of hemp, going from 6 to 12% the T$_0$ only increases by about 5-6 degrees, while in the case of soy the behaviour is different. An increase in concentration from 6 to 9% results in a significant increase in the T$_0$ which rises by about 40 degrees and then decreases again at higher soy concentrations.

To analyse the functionality of the complex modulus with the protein concentration, G* at a given temperature can be evaluated from the temperature ramp test and plotted against the protein concentration for both soy and hemp protein solutions. As it can be observed from Figure 4.6a-b, the complex modulus G* at a 25 °C changes with the protein concentration and seems to linearly increase with increasing the protein content.
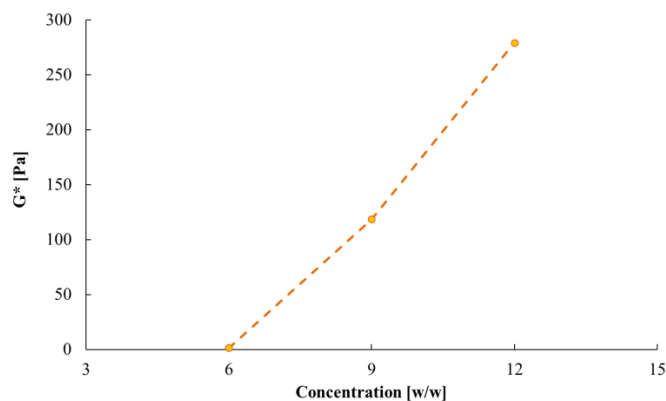
Figure 4.6a   Complex modulus at 25°C vs concentration of soy protein

For the hemp proteins solutions (Figure 4.6b), a different behaviour is observed for G^*. In fact, the complex modulus slightly increases when concentration goes up to 9% while a sharper linear increase is observed at higher concentrations. Probably this behaviour can be attributed to a passage from diluted to concentrated solution, then with different mechanism of aggregation.
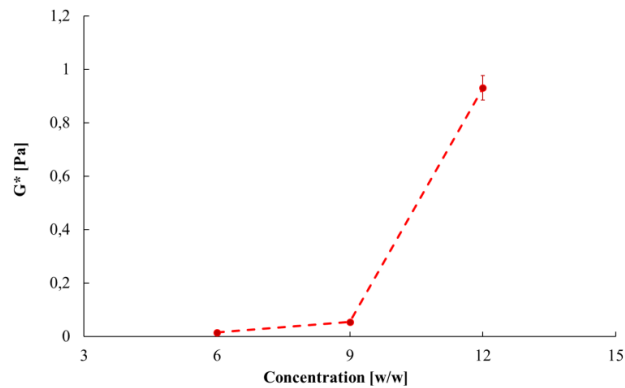
Figure 4.6b   Complex modulus at 25°C vs concentration of hemp protein

4.3.2 Rheological properties of the GF bread model systems

4.3.2.1 Temperature ramp test results

GF control bread

The GF bread model systems, object of study in this work, were obtained by adding hemp and soy proteins at different percentages to a basic bread recipe prepared using only resistant starch as a replacement of a wheat flour. Before analyzing the results for the samples containing the proteins, it's worth showing results obtained for the RS sample which represents the starting point of our study.

The evolution of rheological properties with temperature is shown for RS sample, in Figure 4.7 in terms of storage (G') and loss modulus (G'').
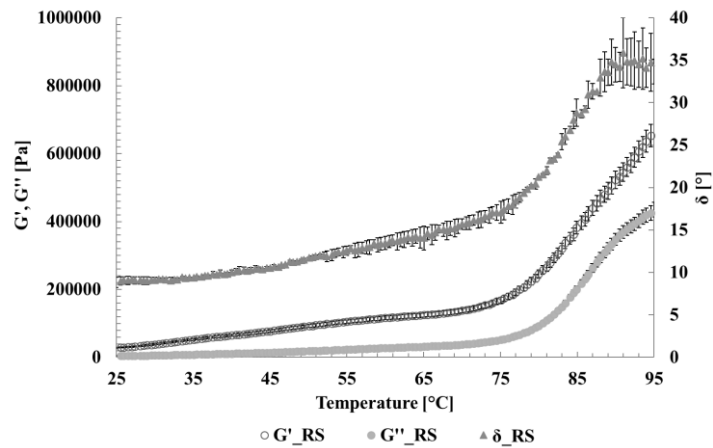
Figure 4.7   G', G'' and phase angle vs temperature for RS dough sample

Increasing the temperature, an initial increase in both loss and storage moduli is followed by a sharp rise probably due to the gelatinization process which is occurring around 70°C, determining an increase in the batter consistency through the formation of a three-dimensional structure (Sanz T. et al, 2008). When heating starch and water based systems, glass transition of the amorphous regions of the starch granules occurs indeed, at a characteristic temperature known as glass transition temperature. Above this temperature, during heating, the amorphous regions are transformed from a rigid glassy to a mobile rubbery state, which corresponds to the gelatinisation phenomena.

The structural changes, that take place during gelatinization, include changes of shape and size of granules, absorption of water and swelling, crystallite melting, and leaching of amylose (amylopectin) from the granules (Jacobs and Delcour 1998). A probably viscoelastic structure formation forms with the thermal structure changes in the RS sample and can be evidenced by an increase in the phase angle with increasing temperature. The T_0 for RS sample, estimated according to Marino et al., 2004, was found of 79.4±5.3 °C.

Effect of protein addition

The effect of protein addition on the temperature dependency of the rheological properties, is shown in Figure 4.8. All the analysed samples with proteins addition shows a similar temperature dependency. Specifically speaking, the complex modulus, G*, increases with incresing the temperature, even if differences in moduli values and critical temperatures were observed.

The mixture with 6% of hemp proteins shows G* values similar to that of the RS sample, while the addition of protein at higher concentrations result in a G* reduction then in an improuving consistency.

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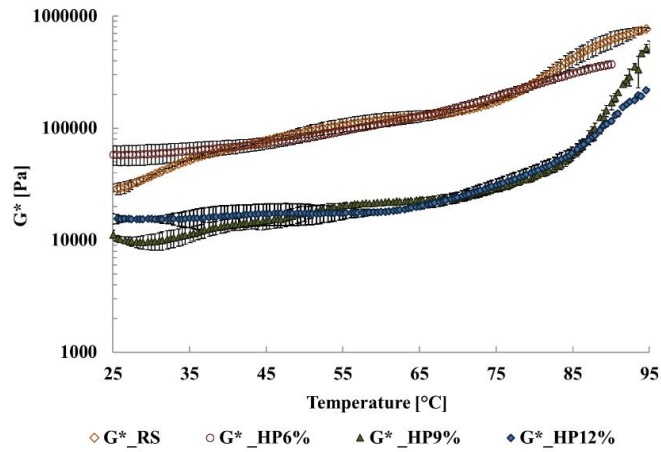
Figure 4.8 Time cure test for RS, HP6%, HP9% and HP12%

Differences in the structuring phenomena occuring in the samples are better evidenced in Figure 4.9 where the phase angles are compared. The phase angle for HP9% and HP12% remains constant up to 70-75°C, not higlighting structuring fenomena below that temperature. For higher temperatures an increase of the phase angle is observed only for HP9% and HP12%, while the HP6% exhibited a different behaviour probably due to the low quantity of protenins not able to give a 3D network.
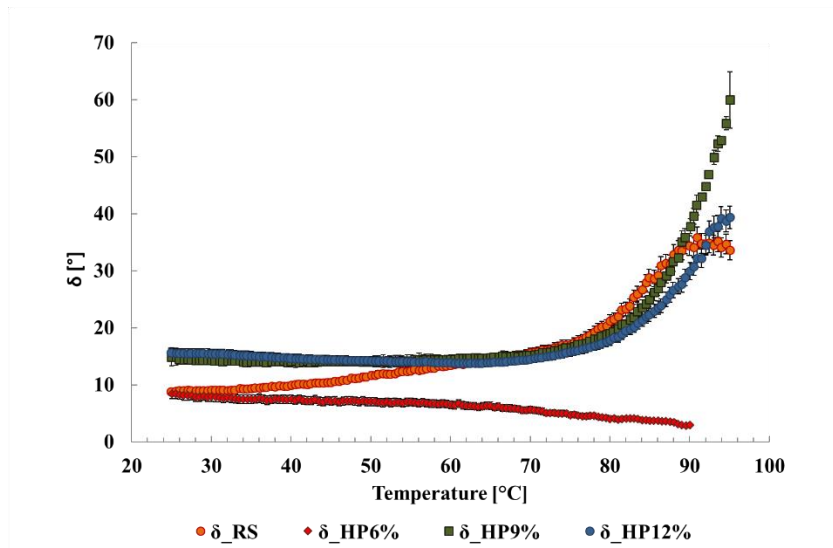
Figure 4.9 Phase angle vs temperature for RS, HP6%, HP9% and HP12%

As far as the soy protein is concenrned, all the samples show G* values higher than the RS benchmark (Figure 4.10). The soy protein-based doughs show a similar temperature dependency with a slight decrease of the complex modulus aroud 40-45 °C followed by a gradual and slight increase at higher temperatures. The addition of the protein to RS, in the case

of soy has a more pronounced effect on the temperature behavior respect to the case of hemp and a reverse effect on the value of the complex modulus compared to the hemp protein addition.

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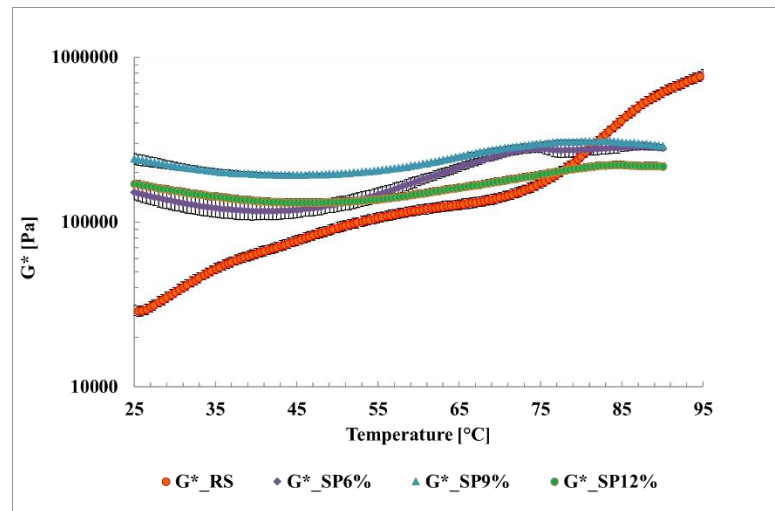
Figure 4.10 Time cure test for RS, SP6%, SP9% and SP12%

In the case of soy, the phase angle did not highlight differences among the samples at different protein concentration.
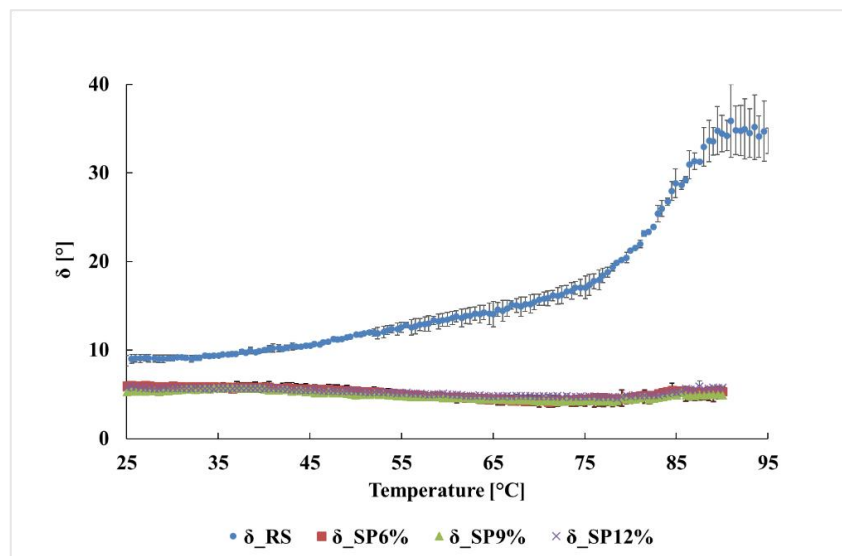
Figure 4.11 Phase angle vs temperature for RS, SP6%, SP9% and SP12%

To compare the different temperature at which transitions occur in the studied GF bread model systems, T_0 was estimated also for these samples accordin to Marino et al., 2014. Results are resumed in Table 4.4.

Samples	T_0 [°C]
RS	79.4±5.3
HP6%	85.0±1.3
HP9%	91.1±4.0
HP12%	71.6±6.0
SP6%	46.1±0.1
SP9%	49.3±1.2
SP12%	50.0±1.0

Table 4.4 T_o for samples ad different soy and hemp protein concentration

Increasing the amount of proteins decreased the T_0 for hemp protein samples. No significant variations of T_0 are observed for the soy protein samples.

The reduction of T_0 with increasing the proteins content in the hemp protein samples, could be justified considering that a high amount of proteins, as reported in literature (Champenois et al. 1998; Jekle et al. 2016), negatively affect the network formation, because of a reduction of the contact between the starch granules thus delaying the starch gelatinization. Protein can build a sort of "barrier" surrounding starch and limiting water diffusion to starch granules and, therefore, hindering the gelatinization. This effect is not related to gluten only and it is observed in other starch-protein systems (Jekle et al. 2016).

The incorporation of high levels of soy proteins has negative effects on the network formation, extensibility properties and gas retention of dough and final bread quality as also reported in other work (Roccia et al. 2009). This behaviour seems to be observed also in the GF analysed samples. Formulations with a lower soy protein concentration exhibit T_0 slightly lower than those exhibited by dough with higher soy protein content. Soy protein produced more resistant, but less extensible mixtures if compared to the hemp.

Effects of mTG-ase addition

The addition of mTG-ase does not significantly affect the rheology in terms of temperature dependency for both the complex modulus G* and phase angle δ. All samples show indeed trends, which are similar to the untreated samples although the addition of the enzyme shifts the transitions occurring in the material because of the starch gelatinization and the protein aggregation. Then, the most interesting parameter remains the onset temperature (T_0) which for the treated and untreated samples is compared in Table 4.5

Samples	T_0 [°C]	Samples	T_0 [°C]
HP6%	85.0±1.3	HP6% TG	59.39±1.54
HP9%	91.1±4.0	HP9% TG	64.28±0.09
HP12%	71.6±6.0	HP12% TG	76.42±0.96
SP6%	46.1±0.1	SP6% TG	64.77±0.91
SP9%	49.3±1.2	SP9% TG	57.09±2.25
SP12%	50.0±1.0	SP12% TG	-

Table 4.5 T_o *for samples at different protein content and treated with m-TGase*

The addition of mTG-ase has an opposite effect on the two types of proteins. As it can be seen from Table 4.5, the enzyme reduces T_o when the hemp protein was added at 9 and 12% while no differences are observed at higher protein content. For samples prepared with soy protein, generally, the T_o increases with the addition of m-TGase and at high soy proteins level T_o is not detectable, probably because the transition is at temperatures outside the range of analysis. A shift towards higher temperature of the transitions detected from the time cure test implies a delay in the hardening of the material structure, allowing the development of the internal bread crumb and the increase of the bread volume. In the case of soy protein, it can be speculated that the m-TGase had a positive crosslinking effect.

According to literature, crosslinked proteins can build a network surrounding the starch and delaying the water diffusion to starch granules and, therefore, hindering the gelatinization. This effect is not related to gluten only and it is observed in other starch-protein systems (Jekle et al, 2016). In the case of hemp, the m-TGase seemed to not have such a positive effect. It indeed anticipates the transitions if compared to the untreated samples behavior. The different effect of the enzyme, as also confirmed in literature, shows that the m-TGase activity varies between the protein type and content (Moore et al., 2006).

4.3.2.2 Frequency sweep test results

Further details on rheological behaviour of considered samples were obtained from frequency sweep tests performed at different temperatures. All the tested samples show a similar trend of G' and G'' in the investigated frequency range.
A typical frequency sweep test obtained from the oscillatory measurements is shown as an example for a quantity of hemp proteins of 12% (Figure 4.12).
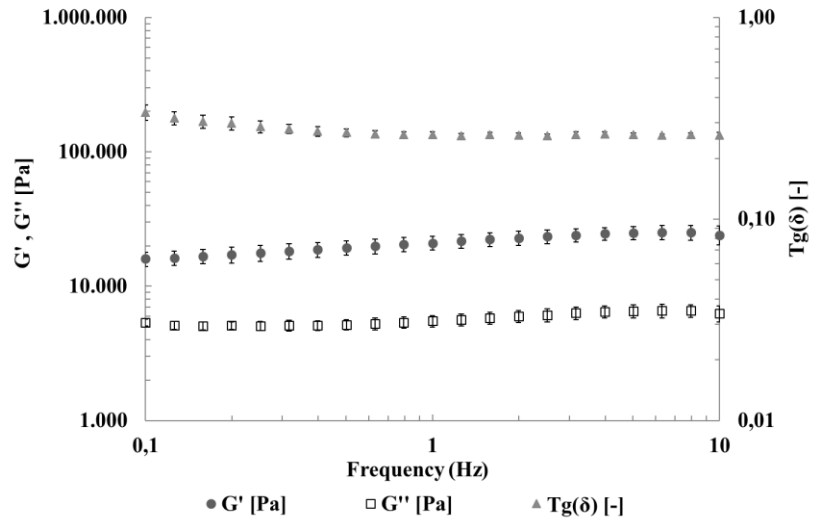
Figure 4.12 Frequency sweep test for HP12% at 25°C

Effect of protein addition

The results are expressed in terms of the weak gel parameters at different temperatures and for all samples and are reported Figure 4.13.
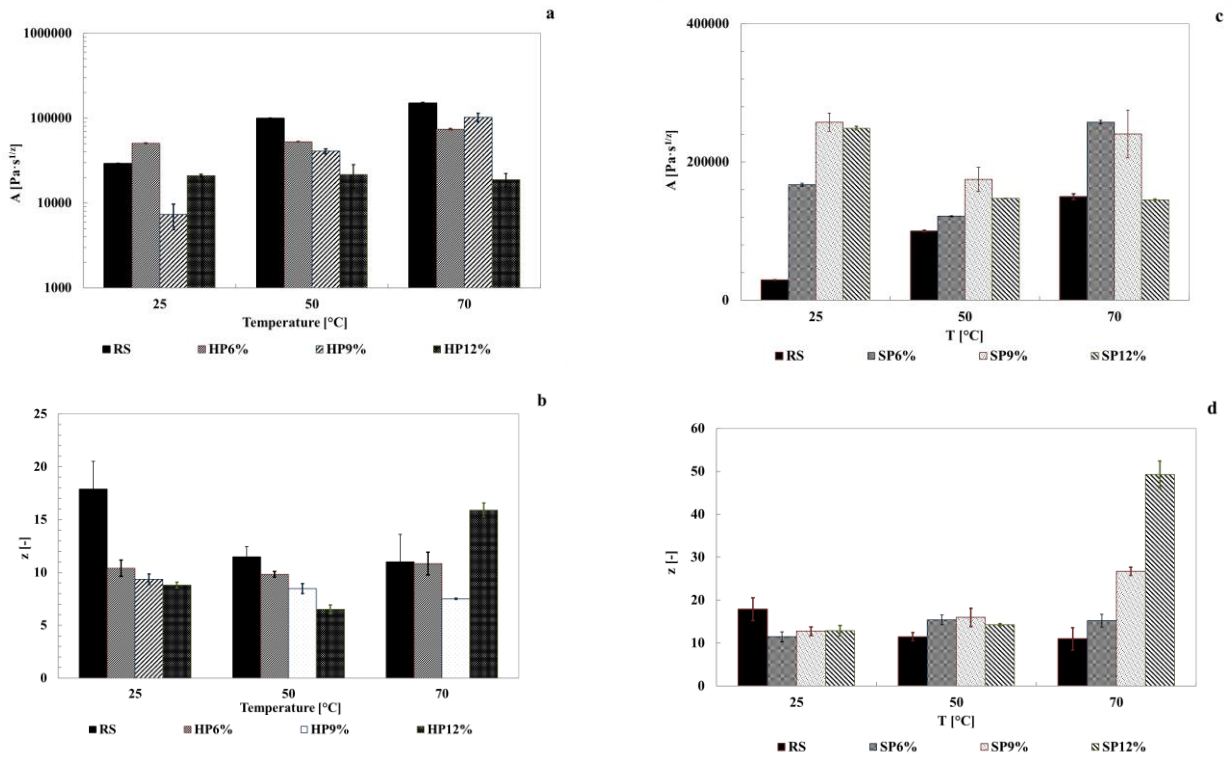
Figure 4.13 Weak gel parameters for dough samples with hemp proteins (a-b) and soy proteins (c-d)

By analysing the parameters of the weak gel model for the untreated samples, it is possible observe that the soy protein gives the most consistent dough, if compared to the hemp. This is evidenced by the larger value of the network strength A (Figure 4.13c); on the other hand, the network extension z is slightly different for the two proteins except for the 12% soy concentration at high temperature.

When temperature increases up to 50°C, the parameter A decreases for all samples prepared with soy proteins, due to the increased mobility of the biopolymers in the system. For the RS an increase in A is probably mainly due the starch swelling; on the other hand, no relevant change in z is observed for RS and all the soy protein samples. Only the SP12% exhibits a differ behaviour in terms of z.

For the hemp protein samples, the temperature does not significantly affect the A value. Differences in the weak gel parameters can be observed for the hemp samples at 25°C and 70°C when changing the concentration of the proteins. For the HP9%, the lower value of the A is observed al the lower temperature and the lower value of z at high temperature, this could suggest the existence of an optimum hemp protein concentration.

Effect of mTG-ase addition

Frequency sweep tests results for treated and untreated samples in terms of weak gel parameters are summarized in Figure 4.14
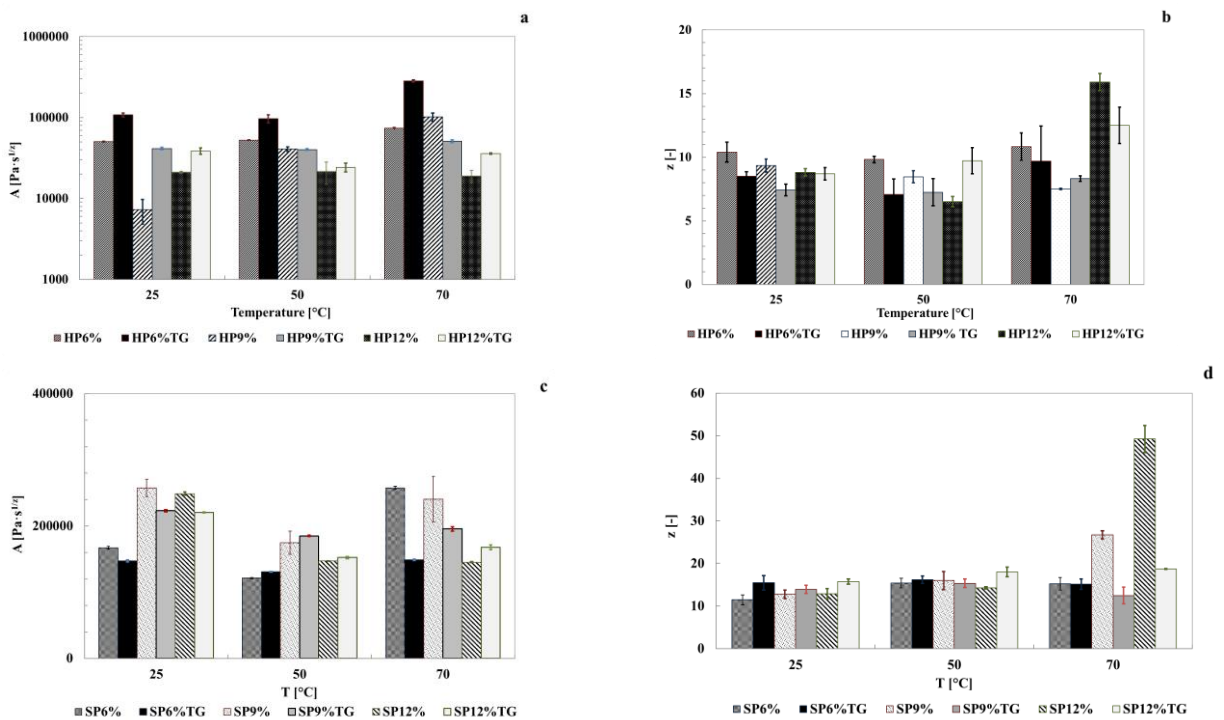
Figure4.14 Weak gel parameters for control and treated samples with hemp proteins (a-b) and soy proteins (c-d)

Adding the m-TGase increases the A value for the hemp protein samples at low temperature and for the HP6% also at higher temperatures. Z value is almost the same for all samples, both for hemp and soy proteins with the exception for SP9%TG and SP12%TG for which z decreased if compared to the untreated sample values.

Adding the m-TGase to soy protein samples, slightly decreased the A value at 25°C. For samples with hemp protein (at 6% and 9%), the gelatinization temperature decreases with the addition of m-TGase as reported in Table 4.5, whereas in the sample with 12% of hemp proteins, no relevant differences were observed between the treated and untreated sample.

4.3.3 Compression test results

In addition to the rheological analysis, samples were also studied performing lubricated squeeze flow tests. The study of the biaxial flow is important in this kind of systems as it can provide useful information to understand the behaviour of the mixtures during the expansion. Data obtained, are reported in terms of biaxial viscosity η_B as a function of strain rate $\dot{\varepsilon}_B$. The viscosity profiles for all samples are similar. As an example, biaxial extensional viscosity η_B as a function of biaxial strain rate $\dot{\varepsilon}_B$, for samples subjected to lubricated squeezing flow at three different crosshead speeds (0.1 mm/s, 0.3 mm/s, 0.5 mm/s), is represented in Figure 4.15.
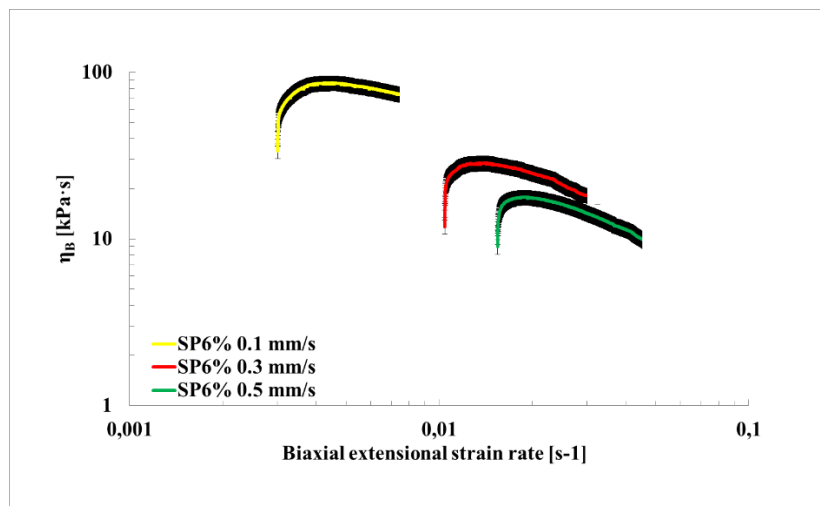
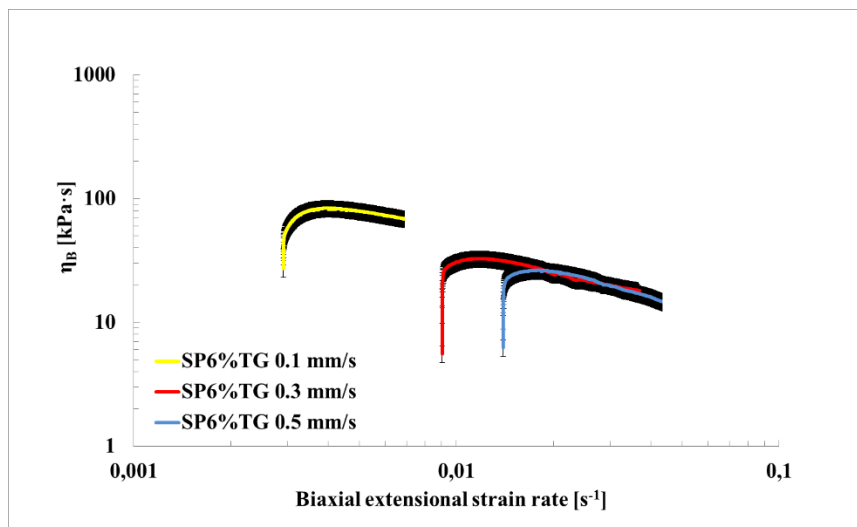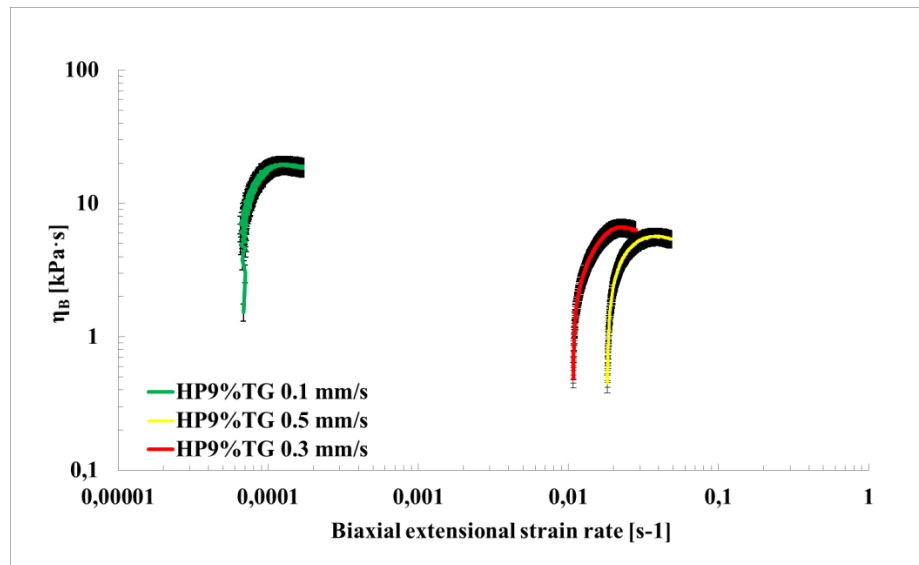*Figure 4.15 η_B as a function of $\dot{\varepsilon}_B$ for the GF dough sample SP6% subjected to lubricated squeezing flow at three crosshead speeds.*

The relationship between biaxial strain rate and apparent biaxial extensional viscosity, in a double logarithmic plot, for sample SP6%, shows a sharp initial rise, followed by a gradual increase in the maximum viscosity and, after that, a slight decrease. Biaxial viscosity decreased with increasing strain rate. The majority of soy protein samples show this marked pseudo plastic

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behaviour (Figure 4.15) while the hemp protein samples showed a less dependency of the biaxial viscosity from the strain rate as reported an example in Figure 4.16. This dependency of biaxial viscosity from the biaxial extensional strain rate is similar to those of glutenin dough reported by Song and coworkers (Song et al., 20088).
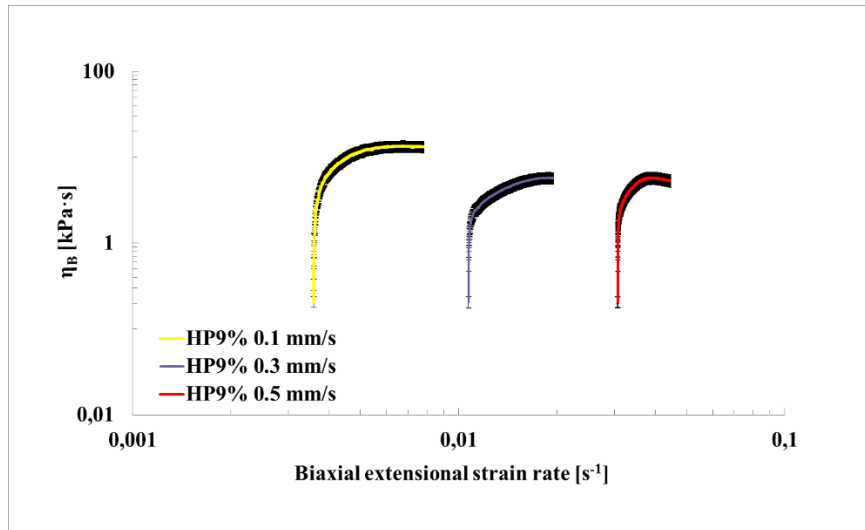
Figure 4.16 η_B as a function of $\dot{\varepsilon}_B$ for the GF dough sample HP9% subjected to lubricated squeezing flow at three crosshead speeds

In Figure 4.17 is compared the behaviour of samples at different soy protein concentration and at the same crosshead speed. As it can be seen, the biaxial viscosity increases when the protein concentration increases.
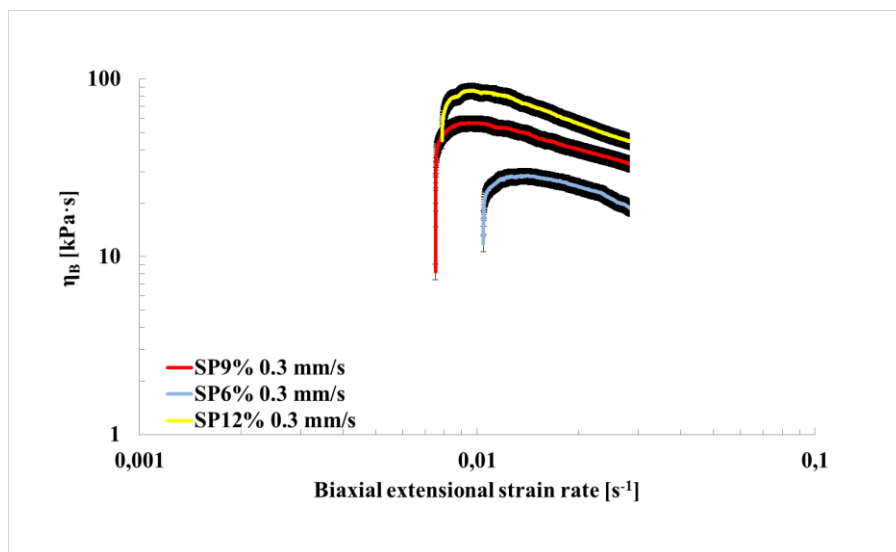
Figure 4.17 η_B as a function of $\dot{\varepsilon}_B$ for samples prepared with soy proteins added at different level, subjected to lubricated squeezing flow at a compression speed of 0,3 mm/s.

For hemp protein samples an increase in the protein content from 6% to 9% seems to increase the biaxial viscosity. For higher concentrations, no differences are observed as shown in Figure 4.18.
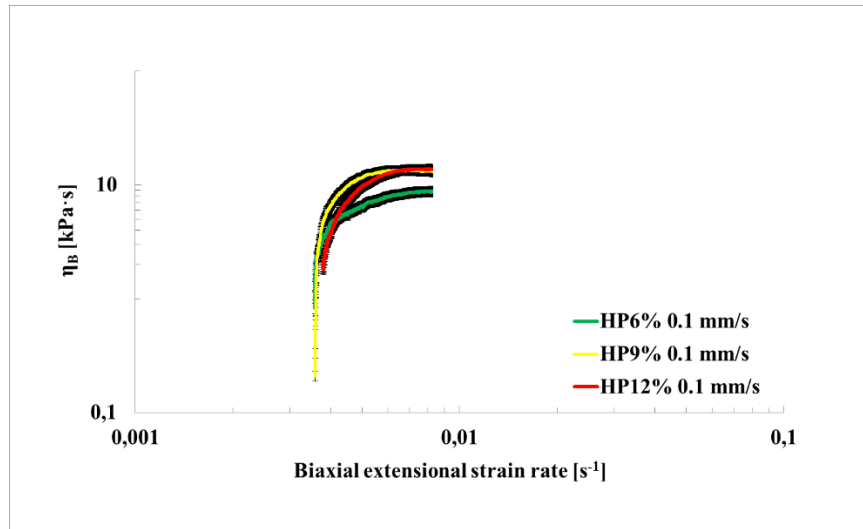
Figure 4.18 - $\boldsymbol{\eta_B}$ as a function of $\boldsymbol{\dot{\varepsilon}_B}$ for samples prepared with hemp proteins added at different level, subjected to lubricated squeezing flow at a compression speed of 0,3 mm/s.

The same pseudo plastic behaviour is observed for soy protein samples treated with m-TGase as shown as an example in Figure 4.19

Figure 4.19 $\boldsymbol{\eta_B}$ as a function of $\boldsymbol{\dot{\varepsilon}_B}$ for the gluten-free dough sample SP6%TG subjected to lubricated squeezing flow at three crosshead speeds

For hemp samples treated with TG, the HP6% TG (Figure not shown) behaves similar to SP6%, (Figure 4.19 above) whereas for higher protein concentrations (for samples HP9%TG and HP12%TG), the addition of m-TGase seems to have induced a slight dependence of the biaxial viscosity from the strain rate as reported in the Figure 4.20, although this is most noticeable for

strain rate values up to 0.01 s^{-1}, while for higher values no relevant variations of the biaxial viscosity are observed.

Figure 4.20 $\boldsymbol{\eta_B}$ as a function of $\boldsymbol{\dot{\varepsilon}_B}$ for the gluten-free dough sample HP9%TG subjected to lubricated squeezing flow at three crosshead speeds.

Comparing the treated and untreated samples at a constant crosshead speed, no differences in terms of biaxial viscosity, at all concentrations, are observed for soy proteins (Figure 4.21). At all the soy concentration samples behaviour was similar to the one reported in Figure 4.21.
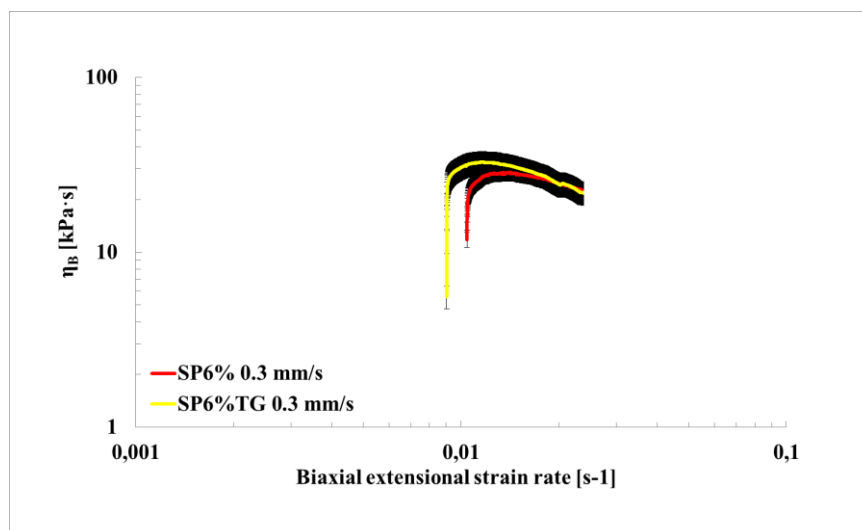
Figure 4.21 $\boldsymbol{\eta_B}$ as a function of $\boldsymbol{\dot{\varepsilon}_B}$ for samples SP6% and SP6%TG, subjected to lubricated squeezing flow at a compression speed of 0,3 mm/s.

For the hemp proteins treated samples at 9% and 12% (Figure not shown) a behaviour similar to the one showed in Figure 4.21 obtained for the soy protein, is observed.

At 6%, the sample treated with m-TGase shows a slightly higher biaxial viscosity if compared to the untreated one as reported in Figure 4.22.
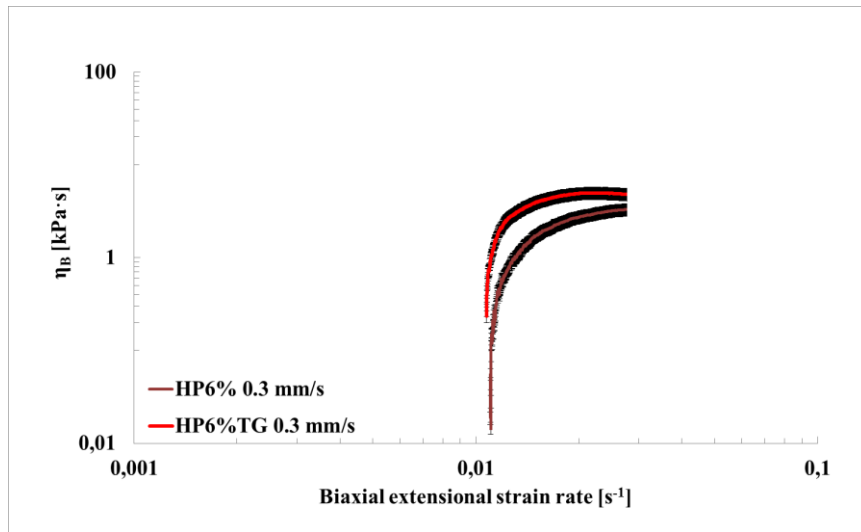
Figure 4.22 $\boldsymbol{\eta_B}$ as a function of $\boldsymbol{\dot{\varepsilon}_B}$ for samples HP6% and HP6%TG, subjected to lubricated squeezing flow at a compression speed of 0,3 mm/s.

As shown in the previous results, $\boldsymbol{\eta_B}$ decreases linearly with increasing strain rate $\boldsymbol{\dot{\varepsilon}_B}$ in the double logarithmic plot, appearing as strain rate thinning behaviour, which can be fitted by the power law model (Rouille et al., 2005):

$$\boldsymbol{\eta_B} = K\dot{\varepsilon}_B{}^{m-1}(4.7)$$

or

$$\boldsymbol{\sigma_B} = K\dot{\varepsilon}_B{}^{m}(4.8)$$

where K is the consistency index and *m* is the power-law index. From literature about wheat dough, macroscopic fractures are usually observed around equibiaxial strain (ε_B) values between 0.35–0.40 (Song and Zheng, 2008). To compare the behaviour of the gluten-free doughs in terms of power law parameters, the resultant values of *m* and K are plotted against ε_B.

In a semi-log plot of K vs ε_B (Figure 4.23a), the HP6% dough consistency (K) seems to slightly increase below $\varepsilon_B \sim 0,3$ while it doesn't change significantly at higher ε_B values. The average K value for the HP9% samples appears to be higher if compared to the other two samples. K seems to not change with increasing ε_B.
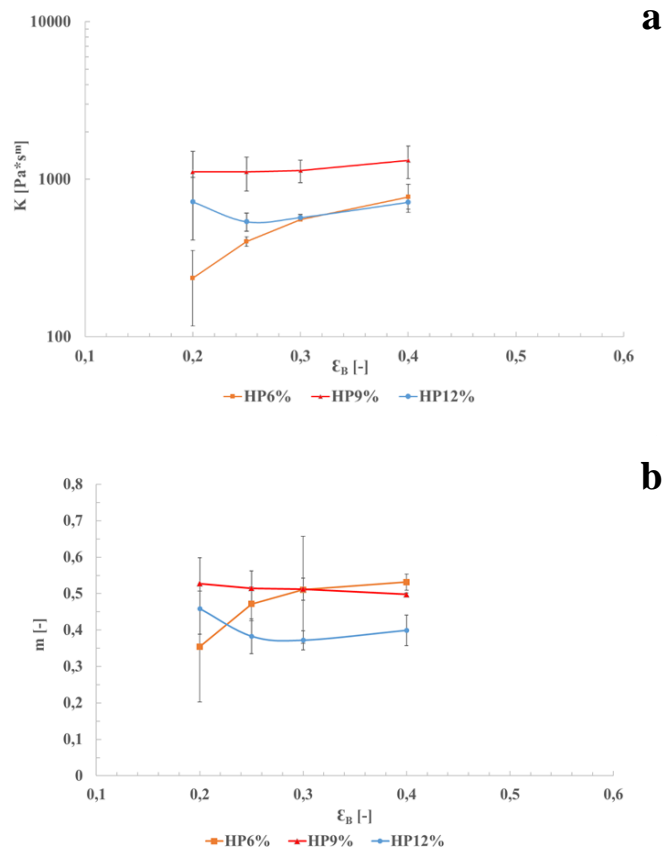
Figure 4.23 a) ***K*** *parameter as a function of* ε_B *for samples HP6%, HP9% and HP12% and R; b)* ***m*** *index as a function of* ε_B *for samples HP6%, HP9% and HP12%*

As far as the power-law index m is concerned, the higher value of m is for the sample with 9% of hemp protein (Figure 4.23b).

A similar behaviour is observed also for the soy protein samples (Figure not shown). Results for these systems resulted more scattered and with a higher standard deviation.

Comparing the results for the treated and untreated sample, no differences in terms of K and m were observed for samples HP6%, HP6%TG, HP12% and HP12TG% (Figure not shown) while differences in the sample at 9% of hemp protein are showed below in Figures 4.24(a-b). From these figures it is possible observe that both the K-value and the m –index decrease with the addition of mTg-ase.
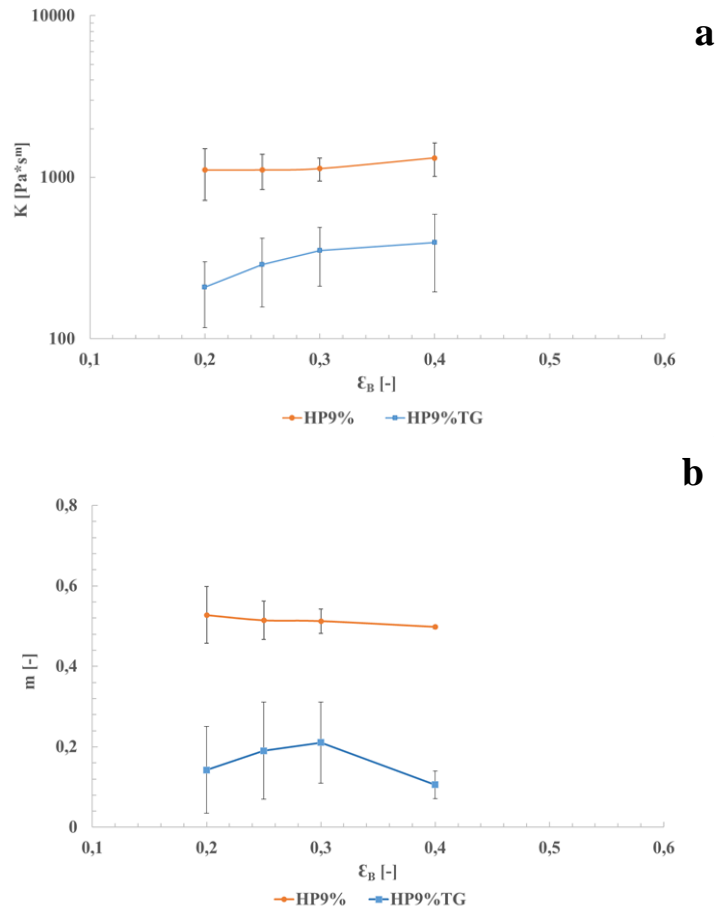
*Figure 4.24 a) **K** parameter as a function of ε_B for samples HP9% and HP9%TG b) **m** index as a function of ε_B for samples HP9% and HP9%TG.*

Although an independency of K from ε_B is observed for both the samples, adding the mTg-ase in the sample at 9% of protein, resulted in a reduction of the consistency of the dough. A completely opposite effect seems to be observed in the soy protein at 9% Figure 4.25.
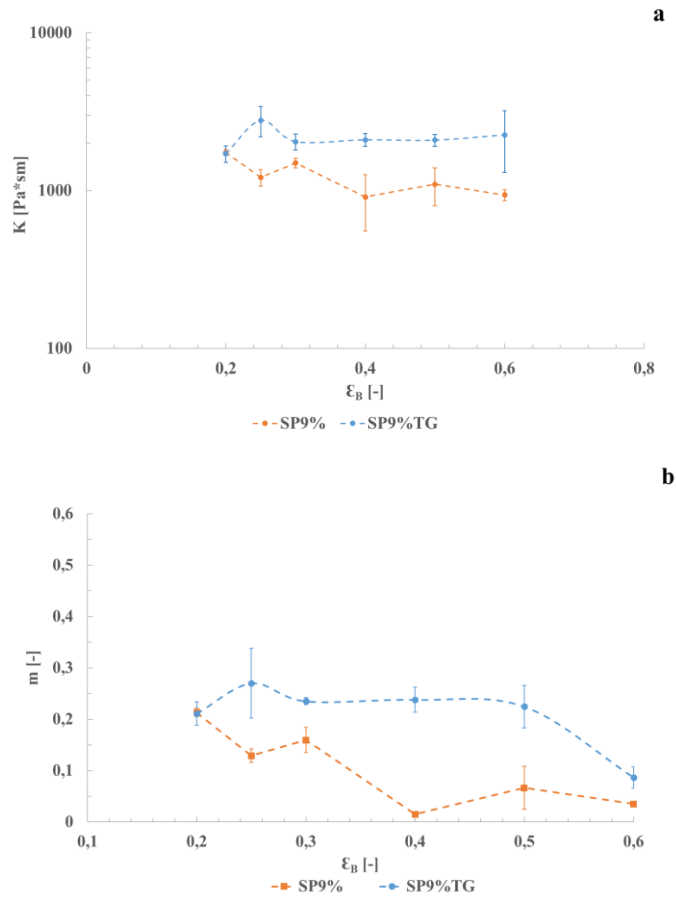
Figure 4.25 a) **K** *parameter as a function of* ε_B *for samples HP9% and HP9%TG b)* **m** *index as a function of* ε_B *for samples SP9% and SP9%TG.*

Samples prepared with soy showed a slight dependency of the consistency K form ε_B index (Figure 4.25a) as also confirmed from the plots of the biaxial strain rate. K and m for the untreated sample HP9% decrease with increasing ε_B while the treated sample shows an independency of the parameters from ε_B but the average value of K and m for the treated sample are higher of the untreated one.

An opposite effect of the m-TGase on the two types of dough prepared with different proteins is also observed from the rheological analysis on the T_0 as reported in Table 4.5 and on the weak gel parameter. The enzyme decreased the T_0 in the hemp protein samples, while increased it in the soy protein samples.

Results of colorimetric analysis of baked products are shown in Table 4.6, in terms of CIELab paramters measured for the bread crumb.

Samples	L* (-)	a* (-)	b* (-)	U w.b. [Kg$_{H2O}$/Kg]	a$_w$ [-]
RS	68.425±5.805	0.341±0.007	0.348±0.007	38±5	0.809±0.033
HP6%	60.775±4.645	0.620±0.735	21.240±3.422	44.95±0.33	0.839±0.001
HP9%	46.040±10.437	2.220±1.923	23.265±3.189	39.87±2.54	0.830±0.007
HP12%	47.34±3.87	2.08±0.01	20.55±0.08	37.06±3.38	0.834±0.001
SP6%	81.265±0.685	0.855±0.940	21.51±6.943	43.76±3.47	0.834±0.015
SP9%	79.215±5.183	0.785±0.516	23.46±10.53	33.82±3.32	0.846±0
SP12%	80.35±2.19	1.125±1.11	22.75±9.899	34.3±3.61	0.852±0.012
HP6%TG	45.035±14.46	2.19±0.61	24.47±0.93	41.04±5.72	0.838±0
HP9%TG	43.505±11.09	1.345±0.064	20.99±2.13	38.06±8.47	0.834±0
HP12%TG	50.49±2.75	1.125±0.106	22.13±0.44	36.08±0.98	0.832±0
SP6%TG	82.67±2.02	1.045±0.487	24.7±8.72	38.38±0.83	0.857±0.004
SP9%TG	83.19±0.42	0.385±0.064	20.16±6.59	41.6±3.28	0.849±0
SP12%TG	80.66±7.03	2.49±3.18	23.52±12.05	32.71±5.00	0.856±0.005

Table 4.6 CIELab paramters measured for the bread crumb

Table 4.6 shows the colorimetric analysis results for both treated and untreated samples. Colorimetirc results, for all the analysed samples, showed differences in terms of degree of brightness (L*), degree of redness (a*) and degree of yellowishness (b*). For control samples prepared with hemp proteins, L* values are lower if compared to results obtained for soy proteins, while higher value of a* and b* for all control doughs are observed if compared to the RS sample. a* and b* also increas as the protein content increases.

Samples treated with m-TGase exhibit a similar behaviour if compared to the untreated control doughs.

In the Table 4.6 are also listed the results for water activity (a$_w$) and humidity (u$_{w.b.}$) measurements for all the analysed samples. For untreated hemp samples, values decrease with increasing concentration of hemp proteins, while for untreated soy samples values increase with increasing the protein content. Both for soy and hemp samples, obtained with the addition of TG, results of of moisture content, similar to the untreated samples are observed, while a$_w$ increases with the addition of m-TGase.

Changing the proteins content, has a clearly affected the bread structure in terms of final loaf and crumb, as shown in Figure 4.26, samples HP6% and HP9% have an internal structure more aerated and soft, although a further improvement of the structure of the crumb is needed.
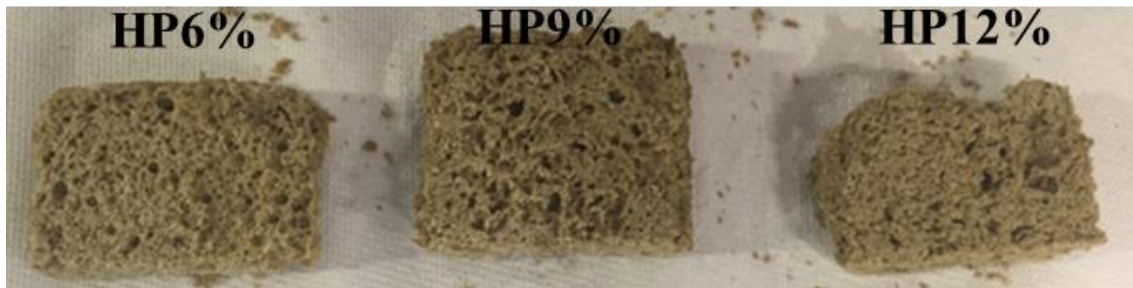

Figure 4.26 Bread loaf comparison for hemp proteins untreated samples

No relevant changes in the crumb and in the loaf volume were observed between the untreated and treated samples, only a slightly smaller size of the bubbles in the treated ones is observed, probably due to the lower T_0 (Figure 4.27).
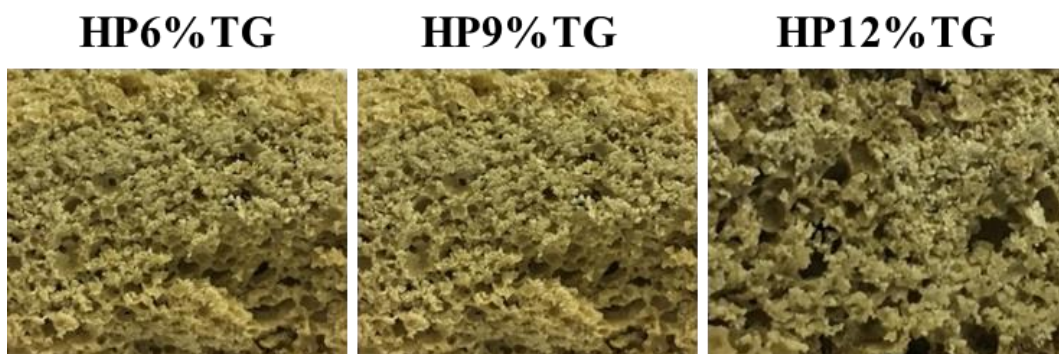
Figure 4.27 Bread crumb of hemp protein samples treated with m-TGase

From a texture point of view, the crumb of the SP6% shows a more aerated structure compared to the others two samples, which appear more compact (Figure 4.28).

Figure 4.28 Bread loaf comparison for soy proteins untreated samples

4.4 Comparison between the glutinic bread and the hemp-protein based model bread

In the light of what has emerged from the results of chapter 4, hemp proteins showed the best baking performance compared to the soy ones, as it can be also observed from the images related to the baking tests. Therefore, hemp appears to be the most promising protein to be used in gluten-free formulations and for this reason the comparison of the hemp protein-based bread with the gluten counterpart bread is presented below to highlight the substantial differences between the two types of product.

From Figure 4.29 it can be observed that the samples at 9% of hemp protein have initial G* values similar to the WF dough, but the thermo-rheological behaviour of the considered gluten free systems is quite different from the WF sample.

The complex modulus of the hemp protein based bread (with and without the enzyme) increases with increasing temperature while in the WF sample, as also discussed in chapter 2, a decrease of G* up to 65°C is followed by an increase of the complex modulus.
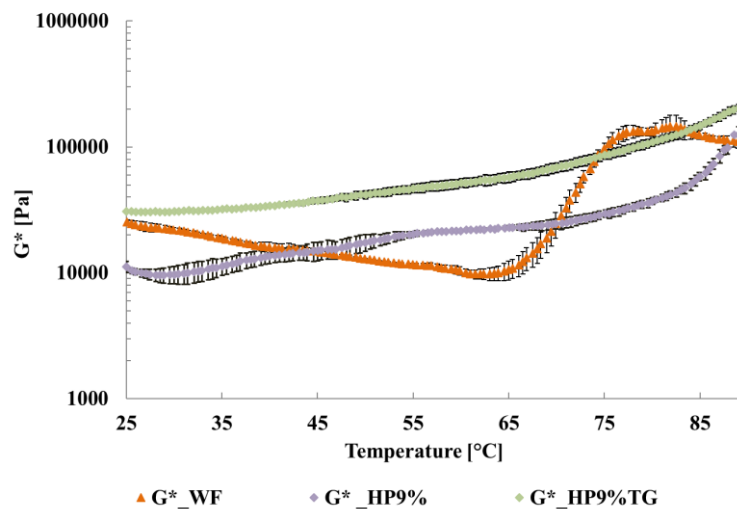
Figure 4.29 Time cure test for WF, HP9%, HP9%TG

The onset gelatinization temperatures are compared in table 4.7

Samples	T_0 [°C]
WF	67.8±0.9
HP9%	91.1±4.0
HP9%TG	64.28±0.09

Table 4.7 Onset temperature of gelatinization for WF, HP9%, HP9%TG

In the WF dough the onset of gelatinization temperature is lower if compared to the HP9% while the treated sample (HP9%TG) exhibit T_o value closer to the glutinic bread, but in despite this results, from the time cure test, in the investigated temperature range, it is not possible to observe for the GF samples, the maximum of the G* modulus which indicates the end of the structuration phenomena. The two types of systems are quite different due to the different type and amount of starch.

The parameters obtained from the extensional data for the three samples, show a similar functionality with ε_B even if the m value is quite different and the independency of m with ε_B, and its higher value, for the HP9%, could be interpreted as a more elastic behaviour of the dough in agreement with the literature (Song and Zheng, 2008), if compared to the treated sample. This is also confirmed by the baking tests when considering the HP9% sample which shows a good expansion of the bread loaf (Figure 4.26) and an internal aerated structure close to a standard wheat flour bread (Figure 4.30)
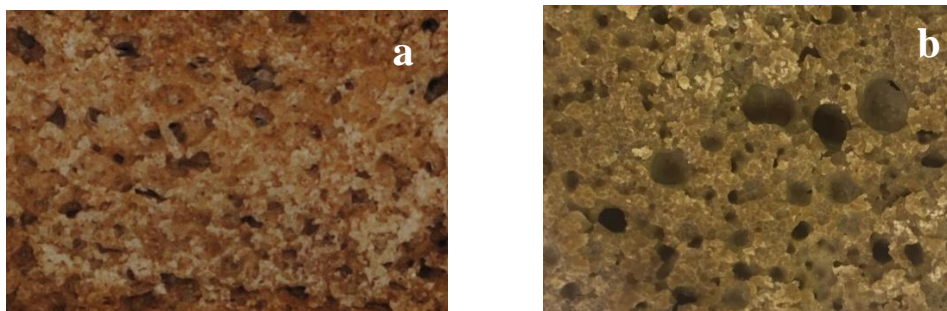
Figure 4.30 Bread internal structure for a wheat flour bread and (a) and HP9% (b)

As a conclusion, it can be observed that these model systems, even if result interesting from a nutritional point of view, they seem to be still far from the rheological behavior of a glutinic ones, confirming the fact that the rheological matching between the two different products is a complex process that needs to be further explored.
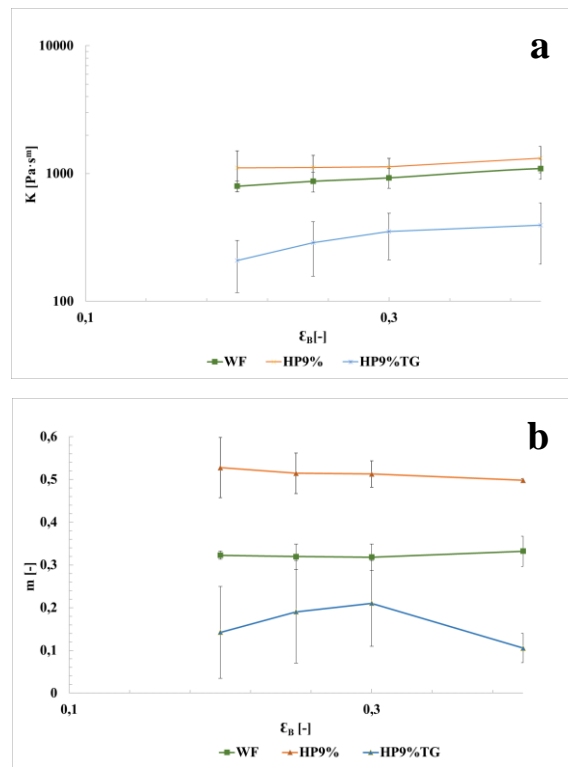
Figure 4.31 a) K parameter as a function of ε_B for samples WF, HP9% and HP9%TG b) m index as a function of ε_B for samples WF, HP9% and HP9%TG.

Conclusions

In this work the effects of the protein addition on a RS starch based dough formulation were investigated with fundamental rheological analysis tests. Two different proteins at three concentrations were used in the formulation prepared using the same amount of water. In GF doughs a higher amount of water leads to a more processable dough with improved final bread characteristics. In the light of what emerged also from the previous study on GF formulations based on buckwheat and rice flour, (discussed in chapter 2) 100% of water (on flour basis) was used for all the samples in this study. Both proteins significantly modified the dough behaviour.

To try to improve the protein structing properties, a microbial transglutaminase was also used to investigate its crosslinking abilities on vegetable proteins such as soy and hemp. The doughs based on soy protein shows good performance at low protein level and the m-TGase addition has effect on the protein crosslinking as confirmed from the rheological analysis in terms of network strength and by the biaxial viscosity.

Opposite effects of the enzyme seemed to be observed in the two proteins. As also confirmed in literature, the m-TGase activity varies between the protein type and content. The addition of m-TGase affected the T_0 of the doughs.

The use of enzyme reduces the T_{0t} in the hemp treated sample and increases it in the soy protein samples then it possible modulate and monitor the properties during baking.

Compression tests show that the soy proteins have a more evident pseudo-plastic behaviour than the hemp protein samples although the hemp proteins led to a better baking performance as it was also observed from the visual inspection. The most prominent effect in the mechanical properties of the dough, is due to the protein content in the soy protein samples.

Obtained results evidence that the use of vegetable proteins at different concentrations can lead to a GF dough more similar to standard wheat bread in terms of aerated internal crumb. In this sense hemp proteins give the best results, but more investigations need to be carried out to further improve the bread structure in terms of volume and softness. In the present work the best results were obtained with 9% of hemp proteins without the addition of the m-TGase.

References

- Bianchi, G. P., Marchesini, G., Fabbri, A., Rondelli, A., Bugianesi, E., Zoli, M., & Pisi, E. (1993). Vegetable versus animal protein diet in cirrhotic patients with chronic encephalopathy. A randomized cross-over comparison. *Journal of internal medicine*, *233*(5), 385-392.

- Björck, I., Eliasson, A. C., Drews, A., Gudmundsson, M., & Karlsson, R. (1990). Some nutritional properties of starch and dietary fiber in barley genotypes containing different levels of amylose. *Cereal Chem*, *67*(4), 327-333.

- Bloksma, A. H. (1990). Dough structure, dough rheology and baking quality. *Cereals Food World*, 2, 35, 237-244.

- Breslau, N. A., Brinkley, L., Hill, K. D., & PAK, C. Y. (1988). Relationship of animal protein-rich diet to kidney stone formation and calcium metabolism. *The Journal of Clinical Endocrinology & Metabolism*, 66(1), 140-146.

- Brown, I. L. (2004). Applications and uses of resistant starch. *Journal of AOAC International*, *87*(3), 727-732.

- Champenois, Y., M. A. Rao & L. P. Walker (1998) Influence of gluten on the viscoelastic properties of starch pastes and gels. *Journal of the Science of Food and Agriculture*, 78, 119-126.

- Chen, N., Zhao, M., Niepceron, F., Nicolai, T., & Chassenieux, C. (2017). The effect of the pH on thermal aggregation and gelation of soy proteins. *Food Hydrocolloids*, 66, 27-36.

- Costantini, L., L. Luksic, R. Molinari, I. Kreft, G. Bonafaccia, L. Manzi & N. Merendino (2014) Development of gluten-free bread using tartary buckwheat and chia

flour rich in flavonoids and omega-3 fatty acids as ingredients. *Food Chemistry*, 165, 232-240.

- De Kruif, C. G., & Tuinier, R. (2001). Polysaccharide protein interactions. *Food hydrocolloids*, *15*(4), 555-563.

- Fuentes-Zaragoza, E., Riquelme-Navarrete, M. J., Sánchez-Zapata, E., & Pérez-Álvarez, J. A. (2010). Resistant starch as functional ingredient: A review. *Food Research International*, 43(4), 931-942.

- Gabriele, D., de Cindio, B., & D'Antona, P. (2001). A weak gel model for foods. Rheologica Acta, 40(2), 120-127.

- Gerrard, J. A. (2002). Protein–protein crosslinking in food: methods, consequences, applications. *Trends in food science & technology,* 13(12), 391-399.

- Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., & Chauhan, B. (2003). Microbial α-amylases: a biotechnological perspective. *Process biochemistry*, 38(11), 1599-1616.

- Hartman, G. L., West, E. D., & Herman, T. K. (2011). Crops that feed the World 2. Soybean—worldwide production, use, and constraints caused by pathogens and pests. *Food Security*, *3*(1), 5-17.

- Higgins, J. A. (2004). Resistant starch: metabolic effects and potential health benefits. *Journal of AOAC International*, *87*(3), 761-768.

- Huang, W. N., Yuan, Y. L., Kim, Y. S., & Chung, O. K. (2008). Effects of transglutaminase on rheology, microstructure, and baking properties of frozen dough. *Cereal Chemistry*, 85(3), 301-306.

- Jacobs, H. & J. A. Delcour (1998) Hydrothermal modifications of granular starch, with retention of the granular structure: A review. *Journal of Agricultural and Food Chemistry*, 46, 2895-2905.

- Jekle, M., K. Mühlberger & T. Becker (2016) Starch–gluten interactions during gelatinization and its functionality in dough like model systems. *Food Hydrocolloids*, 54, Part A, 196-201.

- Jenkins, D. J., Wolever, T. M., Taylor, R. H., Barker, H., Fielden, H., Baldwin, J. M., Bowling, A.C., Newman, H.C., Jenkins, A.L. and Goff, D.V., (1981). Glycemic index of foods: a physiological basis for carbohydrate exchange. *The American journal of clinical nutrition*, *34*(3), 362-366.

- Jiang, S. T., Leu, S. Z., & Tsai, G. J. (1998). Cross-linking of mackerel surimi actomyosin by microbial transglutaminase and ultraviolet irradiation. *Journal of agricultural and food chemistry*, *46*(12), 5278-5282.

- Keri Marshall, N. D. (2004). Therapeutic applications of whey protein. *Alternative Medicine Review*, 9(2), 136-156.

- Keskin, S. Ö., Sumnu, G., & Sahin, S. (2004). Usage of enzymes in a novel baking process. *Molecular Nutrition & Food Research*, 48(2), 156-160.

- Kokelaar, J. J., Van Vliet, T., & Prins, A. (1996). Strain hardening properties and extensibility of flour and gluten doughs in relation to breadmaking performance. *Journal of Cereal Science*, 24(3), 199-214.

- Korus, J., Witczak, M., Ziobro, R., & Juszczak, L. (2009). The impact of resistant starch on characteristics of gluten-free dough and bread. *Food Hydrocolloids*, *23*(3), 988-995.

- Kristensen, M. D., Bendsen, N. T., Christensen, S. M., Astrup, A., & Raben, A. (2016). Meals based on vegetable protein sources (beans and peas) are more satiating than meals based on animal protein sources (veal and pork)–a randomized cross-over meal test study. *Food & nutrition research*, 60(1), 32634.

- Kuraishi, C., Sakamoto, J., & Soeda, T. (1998). Application of transglutaminase for meat processing. Fleischwirtschaft (Germany).

- Marino, R., Giovando, S., & Gabriele, D. (2014). Effect of tannin addition on the rheological properties of starch-based adhesives. *Applied Rheology*, 24(4), 46138.

- Migliori, M. & Gabriele, D. (2010) Effect of pentosan addition on dough rheological properties. *Food Research International*, 43(9), 2315-2320.

- Moore, M. M., Heinbockel, M., Dockery, P., Ulmer, H. M., & Arendt, E. K. (2006). Network formation in gluten-free bread with application of transglutaminase. *Cereal chemistry*, *83*(1), 28-36.

- Motoki, M., and Seguro, K. (1998). Transglutaminase and its use for food processing. *Trends in food science & technology*, 9(5), 204-210.

- Ng, T. S. K. & McKinley, G. H. (2008) Power law gels at finite strains: The nonlinear rheology of gluten gels. *Journal of Rheology*, 52(2), 417-449

- Norajit, K., Gu, B. J., & Ryu, G. H. (2011). Effects of the addition of hemp powder on the physicochemical properties and energy bar qualities of extruded rice. *Food chemistry*, *129*(4), 1919-1925.

- Nugent, A. P. (2005). Health properties of resistant starch. *Nutrition Bulletin*, 30(1), 27-54.

- Ozturk, S., Koksel, H., & Ng, P. K. (2009). Farinograph properties and bread quality of flours supplemented with resistant starch. *International journal of food sciences and nutrition*, *60*(6), 449-457.

- Peressini, D., Sensidoni, A., Pollini, C. M., Gabriele, D., Migliori, M. & de Cindio, B. (2002) Filled-snacks production by co-extrusion-cooking. Part 3. A rheological-based method to compare filler processing properties. *Journal of Food Engineering*, 54(3), 227-240.

- Renkema, J. M., Lakemond, C. M., de Jongh, H. H., Gruppen, H., & van Vliet, T. (2000). The effect of pH on heat denaturation and gel forming properties of soy proteins. *Journal of Biotechnology*, 79(3), 223-230.

- Roccia, P., P. D. Ribotta, G. T. Perez & A. E. Leon (2009) Influence of soy protein on rheological properties and water retention capacity of wheat gluten. *Lwt-Food Science and Technology*, 42, 358-362.

- Rouillé, J., Della Valle, G., Lefebvre, J., & Sliwinski, E. (2005). Shear and extensional properties of bread doughs affected by their minor components. *Journal of Cereal Science*, 42(1), 45-57.

- Sajilata, M. G., Singhal, R. S., & Kulkarni, P. R. (2006). Resistant starch–a review. Comprehensive reviews in food science and food safety, 5(1), 1-17.

- Sanz, T., A. Salvador & S. M. Fiszman (2008) Evaluation of four types of resistant starch in muffin baking performance and relationship with batter rheology. *European Food Research and Technology,* 227**,** 813-819.

- Singh G., (2010). Soybean Processing and Utilization. *The soybean: botany, production and uses*, 345.

- Song, Y., & Zheng, Q. (2008). Influence of gliadin removal on strain hardening of hydrated wheat gluten during equibiaxial extensional deformation. *Journal of cereal science*, 48(1), 58-67.

- Soskey, P. R., & Winter, H. H. (1985). Equibiaxial extension of two polymer melts: polystyrene and low density polyethylene. *Journal of Rheology*, 29(5), 493-517.

- Tang, C. H., Ten, Z., Wang, X. S., & Yang, X. Q. (2006). Physicochemical and functional properties of hemp (Cannabis sativa L.) protein isolate. *Journal of agricultural and food chemistry*, 54(23), 8945-8950.

- Tang, C. H., Ten, Z., Wang, X. S., & Yang, X. Q. (2006). Physicochemical and functional properties of hemp (Cannabis sativa L.) protein isolate. *Journal of agricultural and food chemistry*, 54(23), 8945-8950.

- Tolstoguzov, V. B. (1991). Functional properties of food proteins and role of protein-polysaccharide interaction. *Food Hydrocolloids*, *4*(6), 429-468.

- Trowell, H. (1976). Definition of dietary fiber and hypotheses that it is a protective factor in certain diseases. *The American journal of clinical nutrition*, 29(4), 417-427.

- Wang, X. S., C. H. Tang, L. Chen & X. Q. Yang (2009) Characterization and Antioxidant Properties of Hemp Protein Hydrolysates Obtained with Neutrase (R). *Food Technology and Biotechnology*, 47, 428-434.

- Wang, Y. Y., K. Norajit, M. H. Kim, Y. H. Kim & G. H. Ryu (2013) Influence of extrusion condition and hemp addition on wheat dough and bread properties. *Food Science and Biotechnology*, 22, 89-97.

- Yokoyama, K., Nio, N., & Kikuchi, Y. (2004). Properties and applications of microbial transglutaminase. *Applied microbiology and biotechnology*, 64(4), 447-454.

Study of GF bread model systems based on resistant starch and vegetable proteins and treated with mTG-ase: Part II

Abstract

In this chapter, following the same approach used in the study of GF bread formulations prepared with rice and buckwheat flour, as discussed in Chapter 3, GF bread model systems, based on resistant starch and vegetable proteins treated with m-TGase, were further analysed using thermogravimetric analysis and FT-IR spectroscopy, with the aim of integrating the information gained from the empirical and fundamental rheological measurements discussed in Chapter 4. Using the same samples, it was also possible to perform porosimetry, using low-temperature nitrogen adsorption techniques, with the aim of elucidating the surface characteristics of these systems in terms of specific surface area. Finally, SEM analyses were carried out on selected samples to further explore the characteristic surface morphology of these systems.

5.1 Introduction

The technological and nutritional potentialities of the resistant starch, blended with vegetable proteins has not been extensively exploited in the field of GF bread-making; in most of the works that have looked into this area, the study of GF bread is not conducted with the aim of designing a new flour with targeted nutritional and technological aspects. Rather, resistant starch is used and studied as a substitute in a certain percentage of other types of flour, while in our case it represents a starting point from which to develop an innovative product from the controlled testing of the properties of two types of vegetable proteins.

Some works exist in the literature that study the rheology of GF bread with the addition of resistant starch and vegetable proteins, as reported in Chapter 1, but the use of thermogravimetric analysis and FTIR are very rare because the application of these techniques has several limitations as explained in Chapter 3. This, however, represents a field not widely explored and, therefore, an area that requires in-depth research.

5.2 Materials and Methods

5.2.1 Sample Preparation

GF-bread doughs were obtained, using the procedure outlined in Chapter 4 (Section 4.2.2, Table 4.2). ~30 mg of the fresh dough was used to run TGA analysis. The dried samples, obtained from the TGA measurements, were subjected to FT-IR analysis, using ~15 mg for each run. Well-dried breadcrumb was subjected to BET surface area analysis. Lab-scale bread was prepared using the fresh dough obtained for thermal analysis. After mixing, the dough (with and without m-TGase) was held at a temperature of 40 °C for 1 hr to prove, before baking in a preheated oven, at 185 °C, for 45 min. After baking and cooling, fresh crumb from each sample was pre-dried in a vacuum oven, at 40 °C and for 24 hrs, for a first sample drying, to reduce the time required for the degas stage of the porosimetry measurements.

5.2.2 Experimental runs

All experimental runs were performed according to the procedures described in Chapter 3, including Thermal Analysis (Section 3.2.2.1), Spectroscopic Analysis (Section 3.2.2.2), Porosimetry (Section 3.2.2.3) and SEM analysis of bread structure (Section 3.2.2.7). All samples were prepared and analysed in triplicate.

5.3 Results and discussion

5.3.1 Thermogravimetric Analysis

All analysed GF samples exhibited showed similar TG traces (Figure 5.1-5.4). Also, in this case, the weight loss can be mainly ascibed to deydration processes, according to Fessas and Schiraldi (2001). The mass loss observed with increasing temperature exhibited a sigmoidal descending trend with a flexus at some intermediate temperature where the water loss rate is at a maximum.

Effects of protein content and m-TGase addition

The TGA traces of the samples prepared with hemp proteins, added at different levels, are compared in Figure 5.1. Changing the amount of protein did not significantly affect the slope of the TGA curve, also the was no noticeable shift to higher or lower temperature in the trace. Slight differences seem to be observed only in terms of final mass loss.

The RS control dough seemed to exhibit the highest mass loss at the end of the heating process, in comparison to the sample containing proteins. Tests were conducted, always using the same sample amount, but it is also possible that this result may have been due to factors

related to the high heterogeneity of the analysed samples, despite efforts to minimise errors by reporting the average of three thermogravimetric runs for each sample. This precaution allows the conclusion that the slight differences observed actually represent a significant result, leading to the assumption that, in some ways, protein addition affects water distribution within the sample.
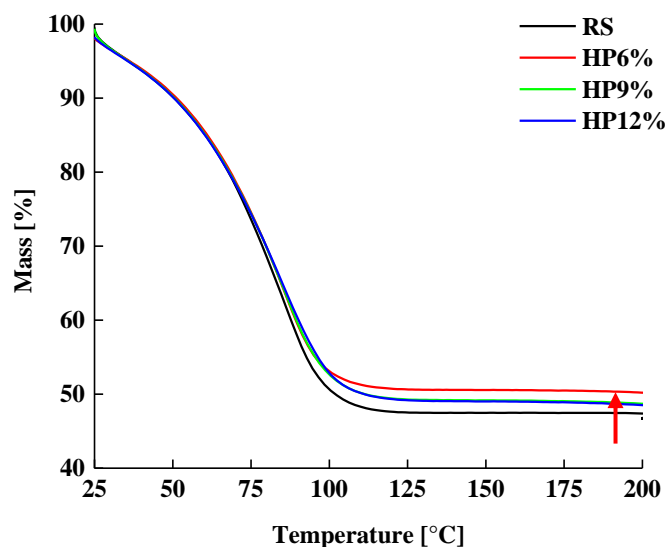
Figure 5.1 TGA curves for samples RS, HP6%, HP9% and HP12%

It was not possible to detect any differences in behaviour for the treated samples using TGA measurements, for addition of m-TGase enzyme, with respect to the case of the untreated samples, at all hemp protein concentrations. This can be seen, as an example, in Figure 5.2, for the dough created using 6% of hemp protein. This is contrast with rheological analysis, which detected some differences in terms of onset gelatinisation temperatures. This result leads to the conclusion that thermal analysis, in this case, is unable to highlight any differences between the considered samples, hence, the temperature range at which the maximum mass loss occurs does not seem to change with the composition of the system.
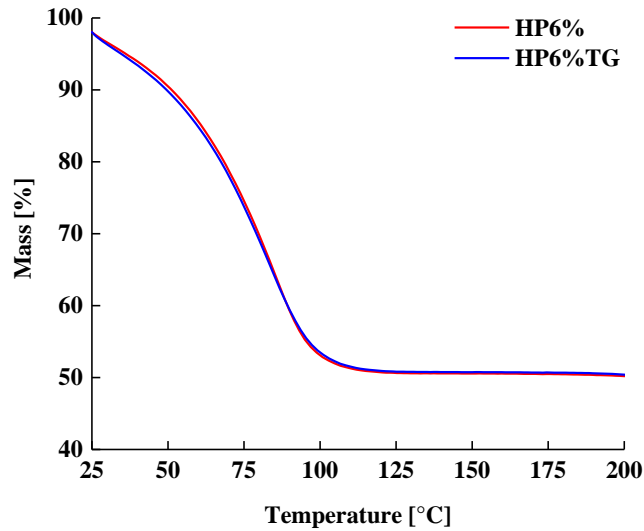
Figure5.2 TGA curves for samples HP6% and HP6%TG

The TGA traces of the samples prepared with soy protein at different levels are compared in Figure 5.3. The same considerations that were made for the samples prepared with hemp are also valid for those prepared at different concentration of soy protein. No relevant differences are detectable between the TG traces when increasing the amount of soy protein.
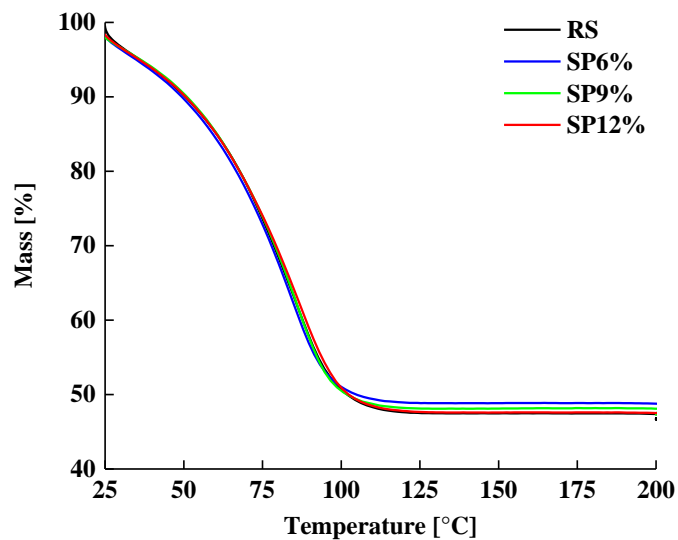
Figure 5.3 TGA curves for samples RS, SP6%, SP9% and SP12%

Adding m-TGase did not significantly affect the behaviour of the sample at 6% and 9% of soy protein, while at the highest protein content a different behaviour is observed, as shown in Figure 5.4.

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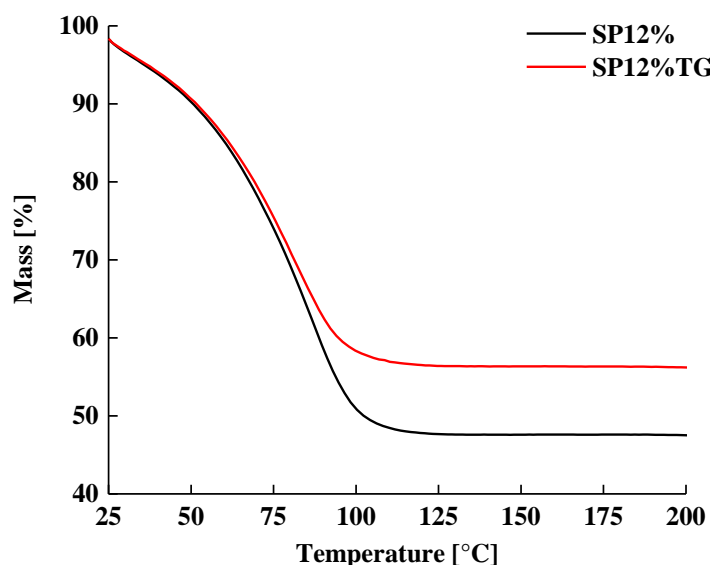
Figure 5.4 TGA curves for samples S96% and SP9%TG

nterpretation of thermogravimetric curves is generally complex and often assumes that some differences, observed in the trends, are actually due to real structural changes in the materials, and should not be undertaken superficially. Also, in this case, the high heterogeneity of the considered material influences the results obtained, and generates some uncertainty in interpreting the data, but such a relevant difference in terms of final mass loss (Figure 5.4) may also suggest that the enzyme has a more pronounced effect on water distribution within the sample in the case of soy protein. This confirms, in some way, the overall results from rheological analysis that m-TGase works better with soy proteins, which from rheological characterisation, was shown to positively influence the onset temperature of gelatinisation, resulting in an increase of this parameter.

5.3.2 Semi-theoretical model for non-isothermal drying kinetics

TGA data were fitted to one of three semi-theoretical models, according to the procedure reported in Chapter 3, Section 3.3.2. The non-isothermal drying models used in this study are derived from isothermal models, and are listed in Chapter 3, Table 3.3. When fitting the moisture ratio (MR) (Chapter 3, section 3.2.2, equation 3.5) to the non-isothermal drying models, an estimation of parameter values is required; non-linear regression analysis was performed using the Levenberg-Marquardt algorithm implemented in OriginPro® 2015. Fitting models were evaluated on the basis of the coefficient of determination (R^2) and reduced chi-square (χ^2). In most cases, the R^2 values for all models are greater than the acceptable R^2 value

of 0.990, indicating good fits. Satistical results, for the three models used are shown in Table 5.1.

Sample ID	Page		Newton		Henderson and Pabis	
	R^2 value (avg)	χ^2 value	R^2 value (avg)	χ^2 value	R^2 value (avg)	χ^2 value
RS	0.9988	1.7E-04	0.9968	4.6E-04	0.9991	1.3E-04
HP6%	0.9987	1.9E-04	0.9962	5.2E-04	0.9989	1.6E-04
HP9%	0.9987	1.7E-04	0.9964	4.6E-04	0.9990	1.3E-04
HP12%	0.9990	1.4E-04	0.9965	4.6E-04	0.9991	1.2E-04
HP6%TG	0.9988	1.6E-04	0.9961	5.0E-04	0.9991	1.2E-04
HP9%TG	0.9989	1.4E-04	0.9963	4.7E-04	0.9991	1.1E-04
HP12%TG	0.9988	1.6E-04	0.9964	4.8E-04	0.9989	1.5E-04
SP6%	0.9983	2.2E-04	0.9959	5.5E-04	0.9989	1.5E-04
SP9%	0.9982	2.3E-04	0.9960	5.3E-04	0.9988	1.6E-04
SP12%	0.9987	1.7E-04	0.9965	4.7E-04	0.9991	1.2E-04
SP6%TG	0.9986	1.9E-04	0.9962	5.0E-04	0.9991	1.2E-04
SP9%TG	0.9969	3.8E-04	0.9962	4.8E-04	0.9991	1.1E-04
SP12%TG	0.9990	1.2E-04	0.9967	1.0E+00	0.9992	1.0E-04

Table 5.1 - Statistical results from non-isothermal drying models

Also, in this case, as for samples prepared with buckwheat and rice flour, based on the χ^2 and R^2value, Henderson and Pabis' model seems to be the best in describing the non-isothermal drying kinetics of these systems. The activation energy (E) for samples prepared with soy proteins, at all soy protein concentrations, is closer to the RS sample, as shown in Figure 5.6, and is ~36 kJ/mol. Slightly smaller values are observed for the samples prepared with hemp proteins (Figure 5.5), while increasing the soy protein content did not affect the value of E; increasing the hemp protein content up to 12% actually resulted in a decrease in activation energy. A reduction in E is also observed in the 12% soy protein sample when adding m-TGase.

The activation energy in these models results from the fit of the dehydration process, and is somehow related to the energy required to remove the water from the samples. The results obtained from the fitting procedures highlight the differences between the two proteins, which, when added to the same starch originally, affect the mobility of water in different ways. Small variations observed from the comparison of the enzyme-treated samples, also confirms that the mTG-ase enzyme affects the structure of the material, even though the effect of this enzyme was not clarified by thermogravimetric analysis.
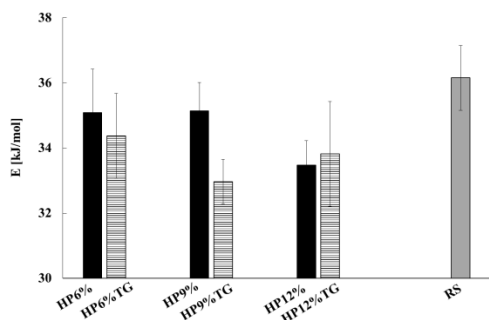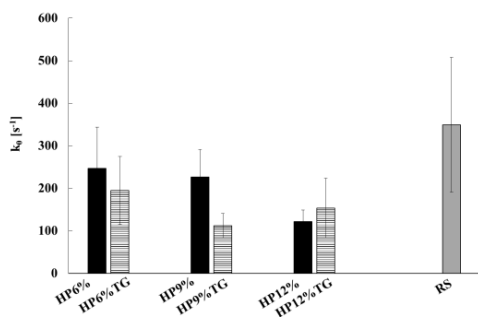
Figure 5.5 Calculated E parameter values of Henderson and Pabis' model for RS, HP6%, HP9%, HP12%, HP6%TG, HP9%TG, HP12%TG
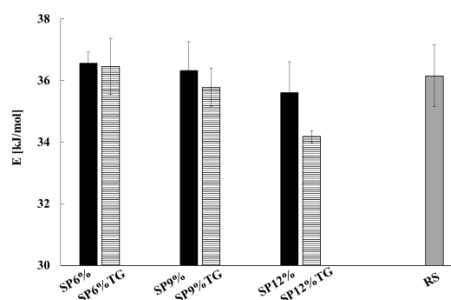
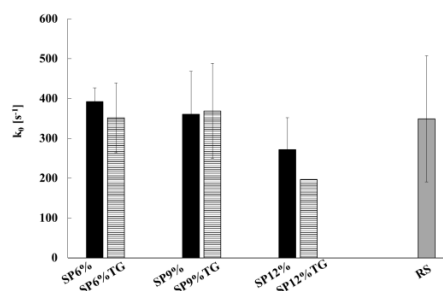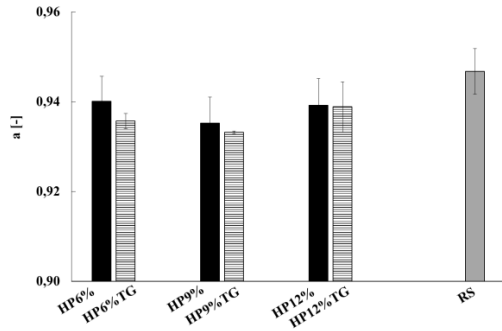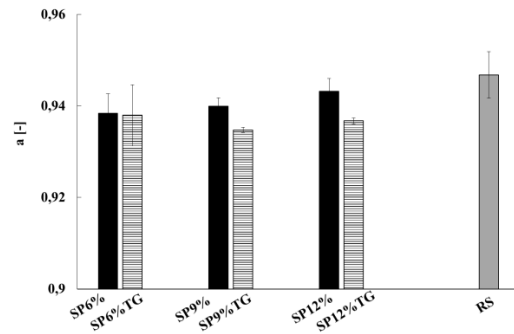*Figure 5.6 Calculated E parameter values of Henderson and Pabis' model for RS, SP6%, SP9%, SP12%, SP6%TG, SP9%TG, SP12%TG*

The Henderson and Pabis model is the first term of a general series solution of Fick's second law (Henderson and Pabis, 1961). The coefficient, k, which in the non-isothermal model is represented by an Arrhenius-like relationship with k_0 as the pre-exponential factor (See Chapter 3, equation 3.7), is related to the effective diffusivity when liquid diffusion controls the process (Madamba et al., 1996). Figures 5.7 and 5.8 show that the two types of proteins differ in terms of their k_0 parameter values, and, in particular, it can be seen that the samples prepared with hemp protein generally have k_0 values smaller than those prepared with soy protein. This also occurs in the case of mTG-ase-treated samples.

Figure 5.7 Calculated k_0 parameter values of Henderson and Pabis' model for RS, HP6%, HP9%, HP12%, HP6%TG, HP9%TG, HP12%TG

Figure 5.8 Calculated k_0 parameter values of Henderson and Pabis' model for RS, SP6%, SP9%, SP12%, SP6%TG, SP9%TG, SP12%TG

No significant differences were observed for the value of the 'a' parameter, as shown for both the hemp and soy proteins, which is ∼ 0.94, while for the RS sample it is slightly higher.

Figure 5.9 Calculated 'a' parameter values of Henderson and Pabis model for RS, HP6%, HP9%, HP12%, HP6%TG, HP9%TG, HP12%TG

Figure 5.10 Calculated 'a' parameter values of Henderson and Pabis model for RS, SP6%, SP9%, SP12%, SP6%TG, SP9%TG, SP12%TG

5.3.3 DTG trace analysis and deconvolution

Thermogravimetric analysis (DGA) of the GF bread model systems with proteins at different incorporation levels were performed to characterise the release of the partitioned water, as previously described by Fessas and Schiraldi (2008), with the aim of gaining more information about the water distribution in these materials, which were not clearly distinguishable from the TG trace. Before discussing the results of the deconvoluted curves, an overview of the DTG traces is shown below. For all analysed samples, the DTG profiles obtained in the present work showed a single broad peak, both in the case of soy and hemp proteins. For the samples made using different hemp protein contents, the DTG traces highlight that increasing the amount of protein results in a decrease in the maximum high of the DTG trace peak (Figure 5.11)
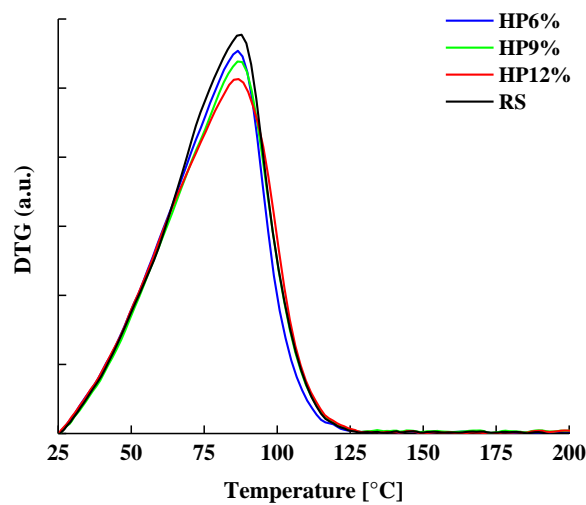

Figure 5.11 DTG traces for RS, HP6%, HP9%, HP12%

No differences were observed in the DTG traces obtained for samples treated with m-TGase (Figure 5.12).
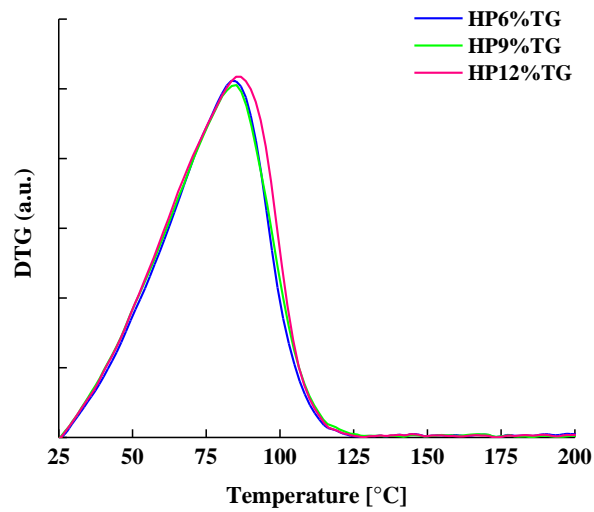
Figure 5.12 DTG trace for HP6%TG, HP9%TG, HP12%TG

In comparison to the results obtained for the 9% hemp protein content samples, in the one treated with m-TGase, the maximum height of the curve is lower when compared to the untreated one.
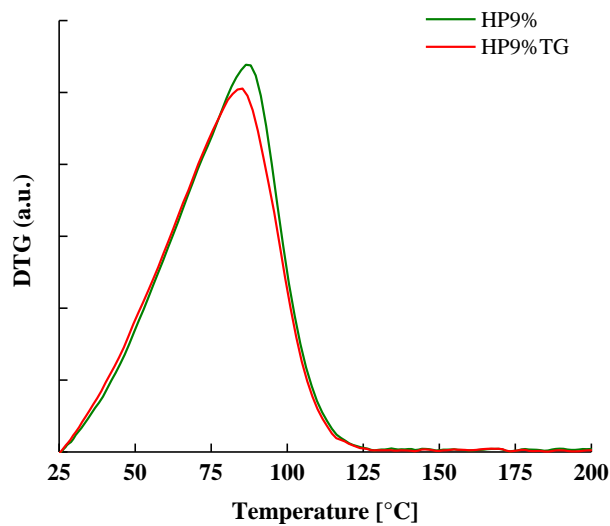
Figure5.13 DTG trace for HP9%TG and HP9%TG

For the samples made using different soy protein contents, the same trend was not observed for the hemp proteins but consideration of the DTG trace highlights that increasing the amount of protein, up to 12%, decreases the maximum height of the DTG trace peak (Figure 5.14).
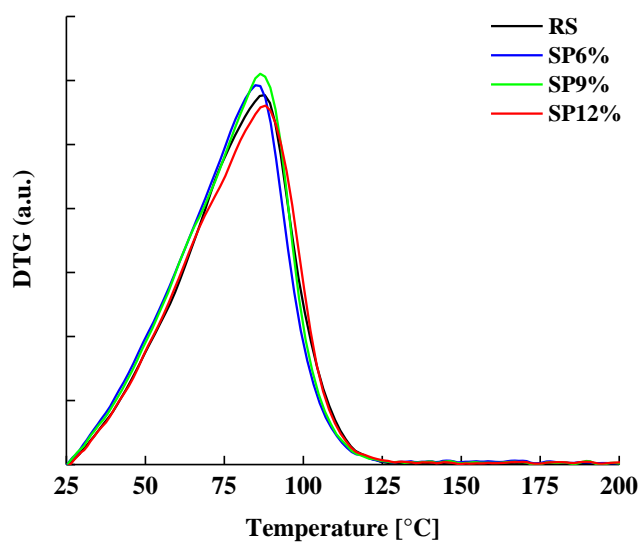
Figure 5.14 DTG trace for RS, SP6%, SP9%, SP12%, SP6%TG, SP9%TG, SP12%TG

For the sample treated with m-TGase, this is also the case at the highest soy protein content, i.e. a reduction in the maximum height of the DTG trace is observed (Figure 5.15).
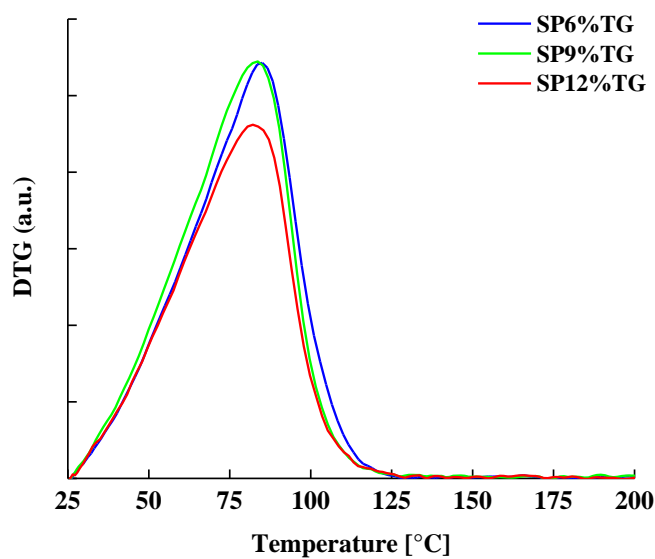
Figure 5.15 DTG trace for SP6%TG, SP9%TG, SP12%TG

The reduction in the peak of the DTG trace is also observed when comparing the samples at 12% soy protein (Figure 5.16).
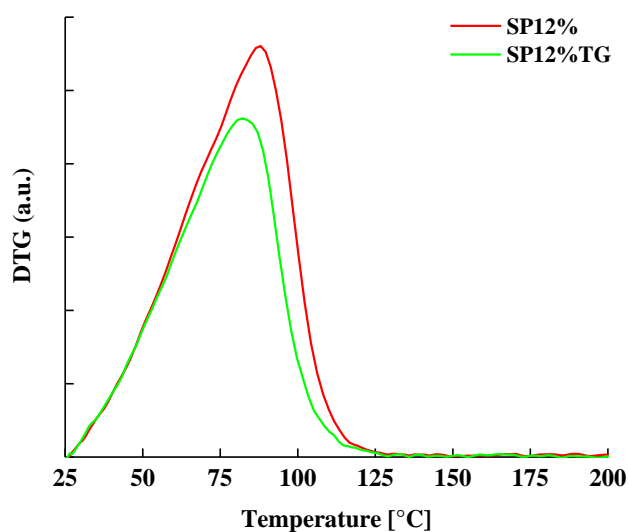
Figure5.16 DTG trace for SP12% and SP12%TG

Results obtained for the GF model system containing resistant starch and vegetable proteins were similar to the DTG traces obtained for GF dough samples prepared with the pseudo-cereals and polysaccharides discussed in Chapter 3. Also, in this case, sample dehydration, described by a single broad peak of the DTG trace, appears to be a single process controlled by moisture diffusion from the core to the surface of the material. According to Fessas and Schiraldi (2008), this should mean that the starch polymers and non-gluten proteins do not compete for water within the system. Accordingly, it follows that, as carbohydrates and proteins are thermodynamically incompatible, the separated aqueous phases could easily exchange solvent between one another, therefore, water evaporating from one aqueous phase is rapidly substituted by water moving from any other nearby aqueous phase. The results for deconvolution of the DTG traces obtained for all samples are shown in Table 5.2.

The results for these GF bread model systems show that there are no significant variations between the analysed samples, both in terms of water distribution and T_{peak}. In general, for most samples, ~73-75% of water represents the Water 1 fraction, and the rest is Water 2. These proportions remain more or less unchanged when increasing the protein concentration, and also when adding the m-TGase enzyme. A slight increase was only observed for Water I in the sample at 12% soy protein. When adding the enzyme, Water I increased from 71±1.2 to 76±1.0 with an analogous decrease in Water 2. A slight decrease in Water I appears to be observed in samples with hemp proteins with increasing protein content. Moving from 6% to 9% hemp protein, Water I decreases from 76±0.8 to 69±3.3 while no relevant results are observed in the samples treated with mTG-ase.

As far as T_{peak} values are concerned, in general, T_{peak1} ranged between 70-72 ºC while the T_{peak2} was between 85-90 °C. In the samples with 9% soy protein, the enzyme reduced T_{peak2} (88±0.4 ºC to 84±2.3 °C) while, in the sample 12% soy protein, T_{peak2} decreased from 90±0.9 ºC to 85±1 °C. In the case of hemp proteins, no differences were observed in T_{peak} for samples at different protein concentrations or for treated samples.

Sample ID	Average water contribution [%]		Average Peak Temperature [°C]	
	Water 1	Water 2	T peak 1	T peak 2
RS	68±1.5	32±1.5	72±1.2	88±0.7
SP6%	73±2.3	27±2.3	71±0.5	87±1.4
SP9%	74±1.6	26±1.6	73±0.5	88±0.4
SP12%	71±1.2	29±1.2	72±0.4	90±0.9
SP6%TG	72±1.2	28±1.2	72±0.2	88±1.3
SP9%TG	74±1.4	26±2.3	69±1.4	84±2.3
SP12%TG	76±1.0	24±1.0	70±0.5	85±1.0
HP6%	76±0.8	24±0.8	72±0.4	88±1.7
HP9%	74±0.8	26±0.8	72±0.4	88±0.7
HP12%	69±3.3	31±3.3	71±1.0	90±0.8
HP6%TG	70±3.0	30±3.0	71±0.7	87±0.4
HP9%TG	62±9.1	38±8.9	68±2.3	88±0.6
HP12%TG	71±0.4	29±0.4	71±0.6	90±1.0

Table 5.2   Water peak distribution and peak temperature of the deconvoluted DTG curves

These results lead to the conclusion that, for these GF bread model systems, the type of proteins used, which are very different from gluten proteins, are not able to strongly modify the water distribution or at least they are unable to modify it, in the samples studied, in such a way as to be detected by thermogravimetric analysis. However, small variations have been observed, but a real trend was not discernible when changing the protein content. As for the addition of enzymes, a major effect was observed in soy protein based samples, especially in terms of T_{peak2}.

5.3.4 Spectroscopic Analysis

Mid-infrared spectroscopy in food analysis

5.3.4.1 FT-IR spectra for bread ingredients

FT-IR absorbance spectra for the major baking ingredients are presented in Figure 5.17.
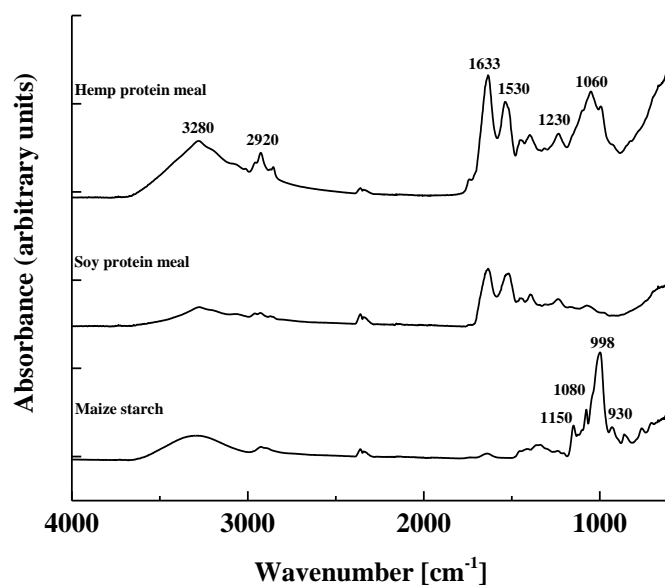

Figure 5.17   FTIR spectra for bread ingredients

All samples were analysed in their dry state; however, all ingredients, showed bands at 2920-3000 cm^{-1} due to C-H stretching modes and a broad peak at 3280-3300 cm^{-1}, due to intermolecular H-bonded and the O-H stretching mode of water (Sivam et al. 2013, Sinelli, Casiraghi and Downey 2008). The spectra for hemp and soy protein meals showed typical protein bands at 1230, 1530 and 1633 cm^{-1}, associated with amide I (originates from C=O stretching with some contribution from N-H vibration), amide II (due to NH bending combined with CN stretching) and amide III (complex band resulting from several coordinate displacements, predominantly due to the in-phase combination of N-H in plane bending and C-N stretching vibration) respectively (Cai and Singh 1999, Barth 2007). The hemp meal spectrum showed an additional prominent peak with a maximum near 1060 cm^{-1}, another characteristic carbohydrate band, mainly related to the presence of native cellulose (C-O vibrations in the cellulose pyranoside units). The soy protein meal does not contain fibres and contains a negligible amount of carbohydrates, as reported in the nutritional data table (Table 5.3), while the analysed hemp meal contains 21% fibre and 15% total carbohydrates. As

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reported in previous work, hemp hurds also contain polysaccharides (Dorado et al. 2001, Garside and Wyeth 2003), therefore, the presence of hemp hurds and other carbohydrates, could explain the additional peak in the hemp meal spectrum, at 1060 cm^{-1}.

In the maize starch powder spectrum, bands originate mainly from the vibrational modes of amylose and amylopectin (Kizil, Irudayaraj and Seetharaman 2002, Pavlovic and Brandao 2003). The analysed sample has intense absorption bands at 900–1200 cm^{-1}, which are characteristic for polysaccharides (Sivam et al. 2013, Cerna et al. 2003, Kacurakova et al. 2000). Performing a deconvolution in this region, allowed resolution of some characteristic peaks found in starches, as shown in Figure 5.18.

	Hemp Protein Meal	Soy Protein Meal
	Per 100g	**Per 100g**
Calories [Kcal]	370	378
Total Carbohydrate [g]	15.3	1
Sugars [g]	5.3	0
Total Fats [g]	12.6	3.3
Saturated Fats [g]	1.3	0.9
Dietary Fibre [g]	21	0
Protein [g]	47	91

Table 5.3 Nutritional data for the hemp protein meal and the soy protein meal

Reduced Chi-Sqr 3,70977E-6
Adj. R-Square   0,99837
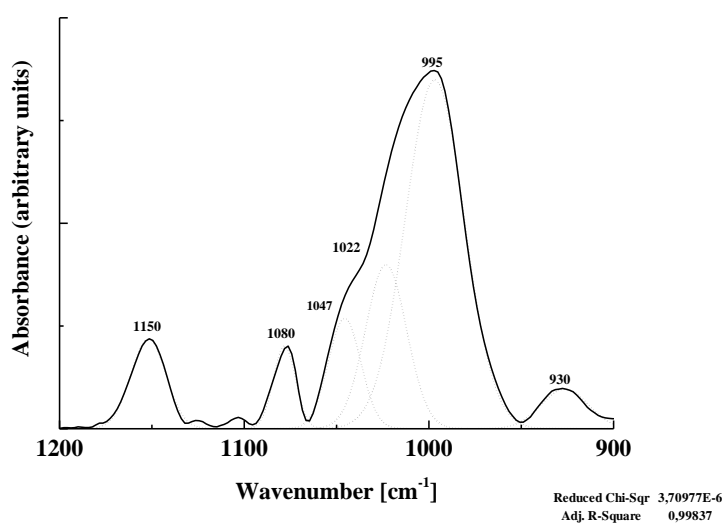
Figure 5.18   Deconvoluted FTIR spectrum for Maize Starch in the region 1200-900 cm^{-1}

In previous work, the infrared spectral region of starch has been described by three main vibrational modes with maximum absorbances at 995, 1022, and 1047 cm^{-1} (Flores-Morales, Jimenez-Estrada and Mora-Escobedo, 2012). Given the complex vibrational modes in the region below 1500 cm^{-1}, which mainly arise from C-C, C-O, C-H stretching and C-OH bending modes, group assignments to specific frequencies, in this region, are highly uncertain (Vasko et al., 1972). However, the band at 1022 cm^{-1}, as reported in previous work (Sevenou et al., 2002), is due to vibrational modes within the amorphous phase of starch. The other band at 1047 cm^{-1} is characteristic of crystalline regions of a starch system (vanSoest et al. 1995, Kacurakova et al. 2000, Kacurakova and Wilson 2001). The band of the skeletal mode vibration of the glycosidic linkage (900-950 cm^{-1}) is observed at 930 cm^{-1} (Kizil et al. 2002). Other characteristic bands of starches, at 1080 and 1150 cm^{-1}, also observed in other types of starches (Liu et al. 2002, Jyothi, Moorthy and Rajasekharan 2006) are associated with the coupled C-O and C-C stretching vibrations of the polysaccharide molecule. Weaker absorption bands are also observed at 1370, 1425 and 1640 cm^{-1}. The peak around 995-1000 cm^{-1} is related to intramolecular hydrogen bonding of the hydroxyl group at C-6, which is highly sensitive to the amount of water in the sample (van Soest et al. 1995)

5.3.4.2 FT-IR for control and treated breads

The FT-IR spectra for the control materials and m-TGase treated samples, are shown in Figures 5.19-5.22.
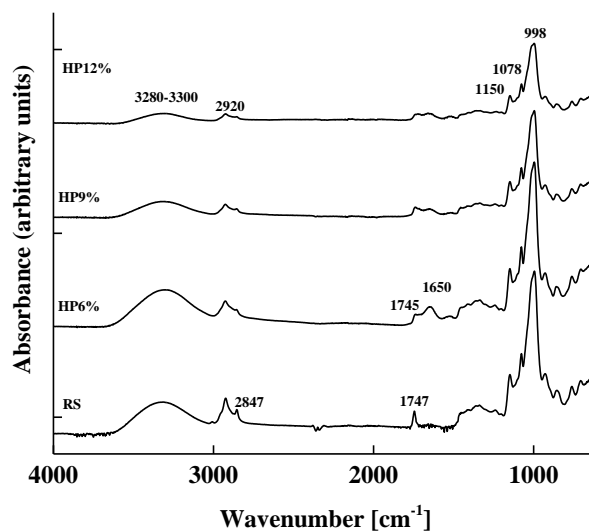
Figure 5.19 FTIR spectra in 4000-550 cm^{-1} for: a) resistant starch (RS) and untreated hemp protein samples (HP6%, HP9%, HP12%);
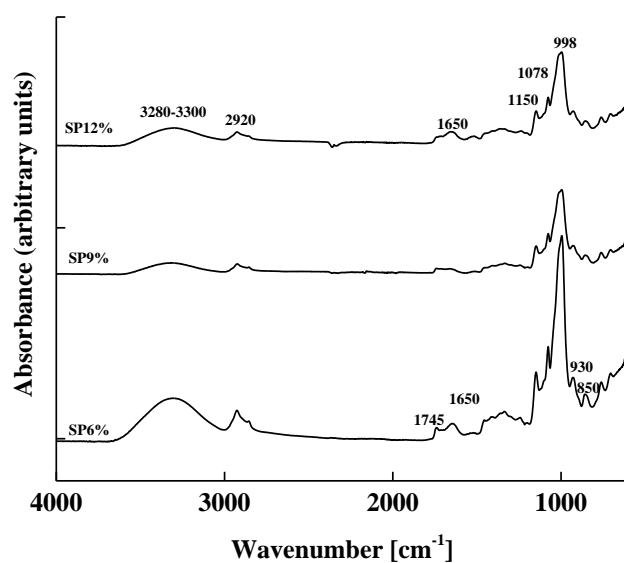
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Figure 5.20 FTIR spectra in 4000-550 cm^{-1} for untreated soy protein sample (SP6%, SP9%, SP12%)
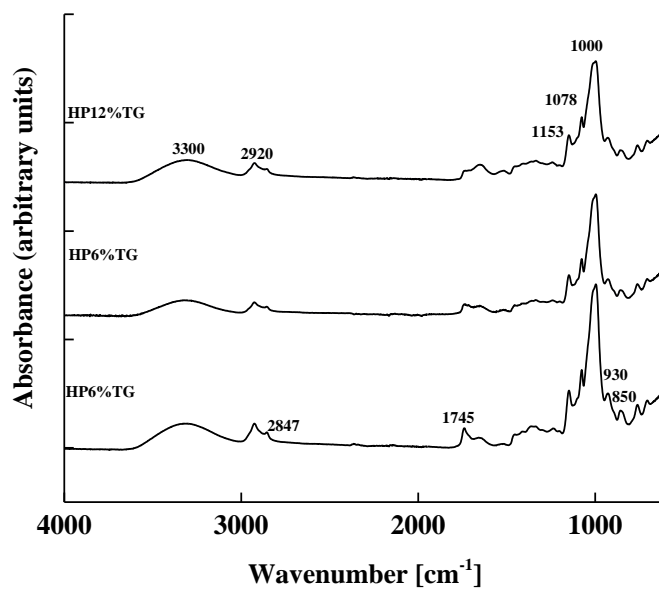

Figure 5.21 FTIR spectra in 4000-550 cm^{-1} for hemp protein sample treated with m-TGase ((HP6%TG, HP9%TG, HP12%TG
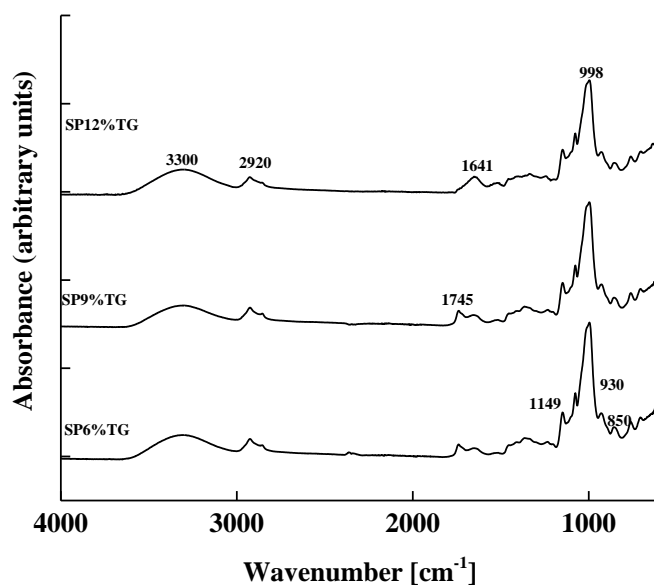
Figure 5.22 FTIR spectra in 4000-550 cm^{-1} for soy protein sample treated with m-TGase (SP6%TG, SP9%TG, SP12%TG)

All samples exhibit similar spectra; the peaks evident for all analysed samples are due to the main components of the dough: water, starch, protein and fat. The peak at 3300 cm^{-1} originates from O-H and N-H bonds (Robertson et al., 2006). The band associated with the O-H stretching mode of water at around 2800-3800 cm^{-1}, in conjunction with the band near 1000 cm^{-1}, assigned to the intramolecular hydrogen bonding of hydroxyl groups, show a reduction in both peaks for increasing protein content in the soy and hemp untreated systems (Figure 5.19-5.20). These effects seem to be less evident in the samples treated with transglutaminase for both hemp and soy proteins, as shown in Figure 5.21 and 5.22. The intensity of the band associated with the O-H stretching seems to be correlated to the amount of water absorbed by the protein polymer (Servatay et al., 2001). This suggests that adding the crosslinking enzyme, could modify the water-protein interaction.

The peak at 1745-1747 cm^{-1} is due to the carbonyl stretching of the fatty ester groups from lipids (van Velzen et al. 2003); its intensity is greater in the RS and HP6%TG samples, which may result from the ether groups and termination of bonding for these groups with temperature. Water tends also to absorb at ~1645 cm^{-1} due to O-H bending, which is also within the amide I region (1600-1700 cm^{-1}). The bands in this particular region of the spectrum are very sensitive to conformational changes in the protein secondary structure.

The region from 1500-1600 cm^{-1} is amide II, wich is primarily N-H bending and C-N stretching modes, and it is particularly sensitive to protein-solvent interaction and protein-protein hydrophobic interaction (Mejia, Mauer and Hamaker 2007). Variations in the line shape of the bands related to the amide I and amide II regions, which are discussed in the secondary protein conformation analysis (Section 3.6.3), occur among the doughs. In the RS sample, the amide I and amide II regions are absent (Figure 5.19), which is to be expected as there are no related functional groups within the protein structure. The region from 800-1300 cm^{-1} (C-C, C-O) is indicative of starch and its hydrolysis products, and this region is not perturbed by water (Kacurakova and Wilson 2001, Kacurakova et al. 2000); however, it is notable that the relative intensity of this region decreases as the degree of substitution increases.

5.3.4.3 Secondary protein conformation analysis

Thermal treatment causes the partial loss of the protein secondary structure, and also leads to protein aggregation. As a consequence of this, as far as FTIR analysis is concerned, some bands are minimised or eliminated, and new bands, related to new molecular arrangements, are formed (Hammann and Schmid 2014). The amide I and II bands are the two major vibrational bands of the protein backbone (Krimm and Bandekar 1986, Kong and Yu 2007).

The amide I band (1600−16700 cm−1) is due almost entirely to the C=O stretching vibrations of the peptide linkages (approximately 80%), which are modulated by the protein secondary structure (α-helix, β-sheet, etc.) (Robertson, Gregorski and Cao 2006). It is the spectral region that is most sensitive to protein secondary structural components (Hammann and Schmid 2014). The amide II band, in contrast, derives mainly from in-plane N-H bending (40-60% of the potential energy) and from the C-N stretching vibration (18-40%), showing much less protein conformational sensitivity (Kong and Yu 2007).

Curve fitting procedures were applied to the FTIR spectra of control and treated samples, over the region of 1500 -1800 cm^{-1}. More attention has been given to the amide I region, to interpret the structural chages occurring in the samples upon heating. Due to its low signal, the amide III region (1295 - 1330 cm^{-1}) has been neglected in this study.

Soy Protein

The deconvoluted amide I and amide II regions, for all the analysed samples, consist of overlapping component bands, representing α-helices, β-sheets, turns and random structures. For both the control (Figures 5.23-5.24-5.25) and treated (Figures 5.26-5.27-5.28) samples, the amide I band is centered around 1650 cm^{-1}, exhibiting an asymetrical shape and the amide II band is centered around 1520 cm^{-1}. A splitting of the peak in the amide I region can be observed for both SP6% (Figure 5.23), and SP9% (Figure 5.24), but is more evident in the sample containing 9% soy protein. The deconvoluted amide I band for the SP6% sample showed a highly intense band at 1650 cm^{-1}. Components centered between approximately 1658 and 1650 cm^{-1} have been assigned to the α-helix (Kong and Yu 2007, Chirgadze and Nevskaya , 1976).

Weaker peaks are observed around 1622, 1630 and 1636 cm^{-1}, where bands in the regions of 1620−1640 cm^{-1} and 1695−1690 cm^{-1} can be assigned to β-sheet conformation, as has been previously reported by many authors (Barth 2007, Kong and Yu 2007). Strong intermolecular interactions have also been considered to be responsible for the bands around 1620 cm^{-1} (Barth, 2007); the appearance of a low-frequency band below 1620 cm^{-1} but also between 1620-1625 cm^{-1} seems, in fact, to originate from the formation of intermolecular hydrogen-bonded antiparallel β-sheet structures at the expense of other regular structures (α-helix, β-sheets) (Torii and Tasumi 1992, Matheus et al., 2006). Another minor peak around 1670 cm^{-1} (β-turns) is observed; while bands around 1670, 1683, 1688 and 1694 cm^{-1} are usually assigned to β-turns (Kong and Yu 2007, Barth 2007) which are known, on an experimental basis, to give rise to the amide I bands in a wide wave number region (1660-1695 cm^{-1}) this overlaps with the higher wavenumber band of β-sheets, making the assignments difficult (Torii and Tasumi 1992). Peaks at 1522, 1540 and 1556 cm^{-1} (β-turns) were deconvoluted from the amide II region and can be ascribed to secondary amide bending modes.

Increasing the amount of soy protein to 9%, reduced the overall intensity of the amide I band (Figure 5.24), showing a less dominant α-helix structure (1657 cm^{-1}). An additional weak band around 1607 cm^{-1}, assigned to β–turns, (Hammann and Schmid, 2014) can be observed. No peaks assignable to intermolecular β-sheets can be observed in the SP9% sample. Increasing the soy protein content up to 12% (Figure 5.25), led to dominant β-turns (1666 cm^{-1}) and β-sheets (1642 cm^{-1}), as shown in Figure 5.25 a minor peak around 1685 cm^{-1} (β-sheets) is also observed. The peak around 1619 cm^{-1} (intermolecular β-sheets conformation) also appears for the SP12% sample.
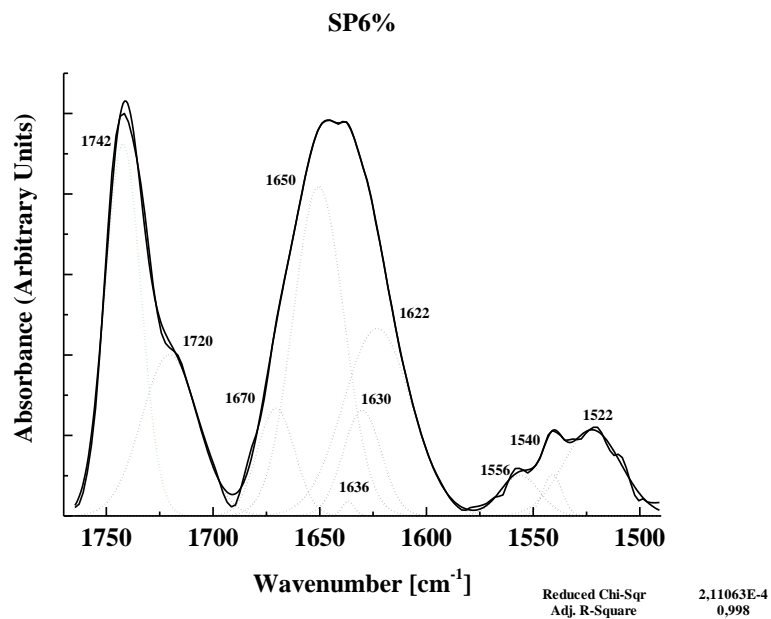
SP6%

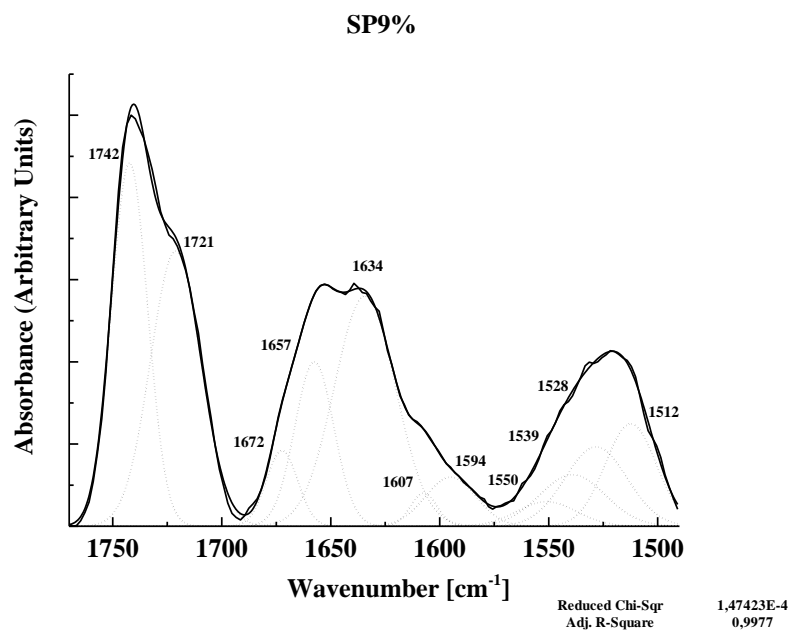*Figure 5.23 FTIR spectra in the 1800-1500 cm⁻¹ range for SP6%*

SP9%

Figure 5.24. FTIR spectra in the 1800-1500 cm⁻¹ range for SP9%

190

SP12%

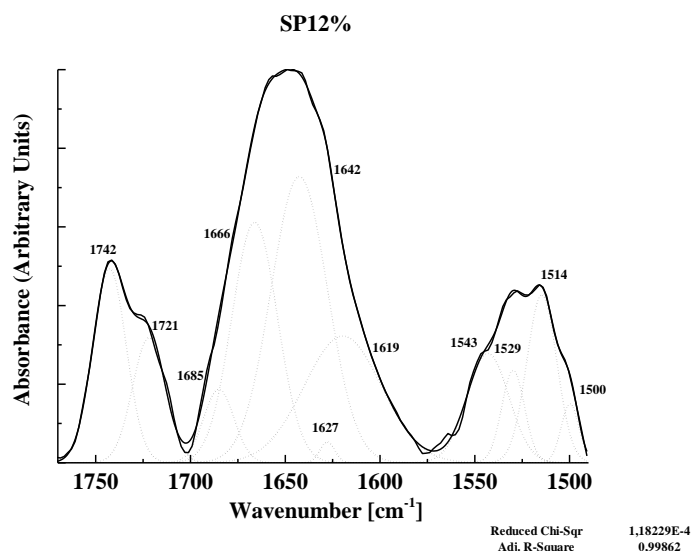|	
Reduced Chi-Sqr	1,18229E-4
Adj. R-Square	0,99862

Figure 5.25 FTIR spectra in the 1800-1500 cm⁻¹ range for SP12%

Adding the enzyme reduces the α-helix signal intensity for both SP6%TG (1652 cm^{-1}) and SP9%TG (1653 cm^{-1}); at the higher protein content, the enzyme induces a reduction of the β-turns conformation (1672 cm^{-1}) and a β-sheet predominant conformation (1642 cm^{-1}). The band, which could probably be assigned to intermolecular β-sheets, seems to be present in the amide I region, only for the SP6%TG sample (Figure 5.25) around 1618 cm^{-1}. Peaks around 1500-1510 in the amide II region are also associated with the presence of extended and intermolecular β-sheets bonding. These can be observed in the SP9%TG and SP12%TG samples.

SP6%TG

Reduced Chi-Sqr	3,67883E-5
Adj. R-Square	0,99934
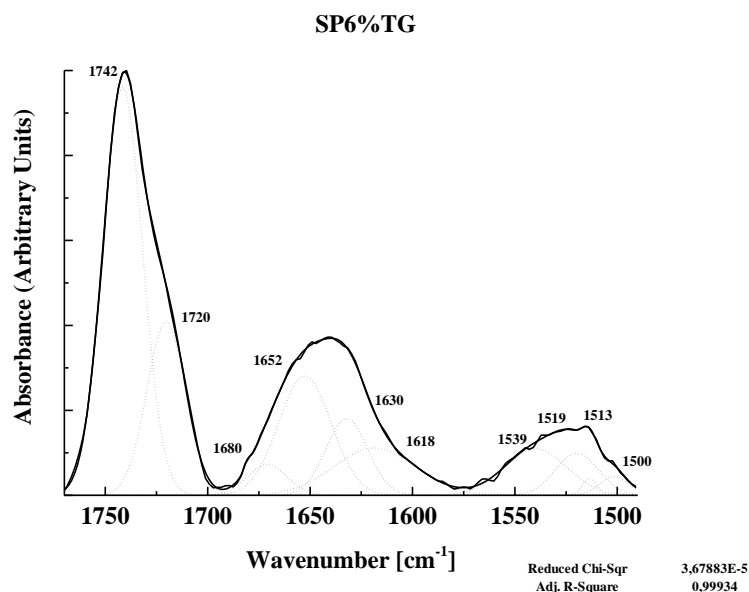
Figure 5.26 FTIR spectra in the 1800-1500 cm⁻¹ range for SP6%TG

SP9%TG

Reduced Chi-Sqr   1,71668E-5
Adj. R-Square   0,9997
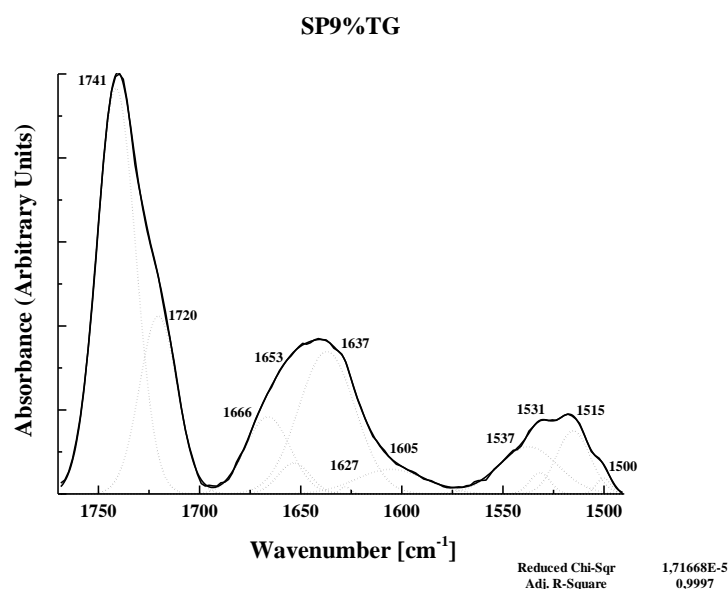
Figure 5.27 FTIR spectra in the 1800-1500 cm^{-1} range for SP9%TG

SP12%TG

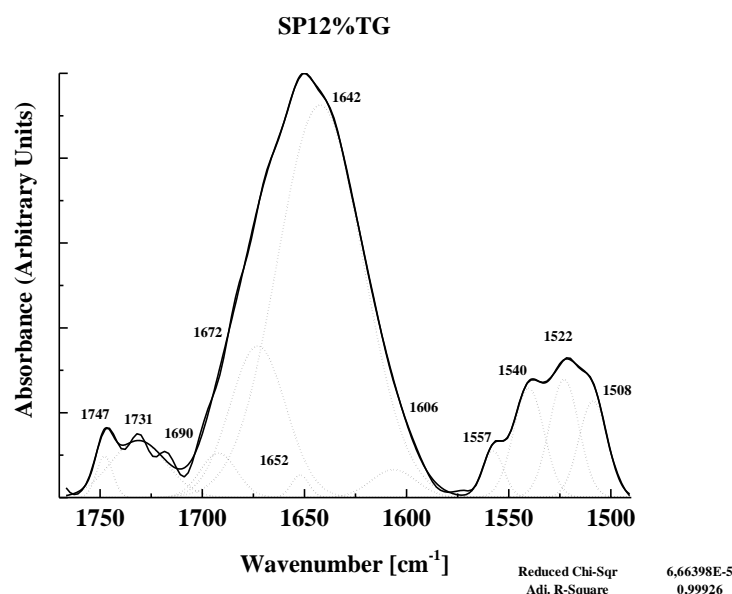Reduced Chi-Sqr   6,66398E-5
Adj. R-Square   0,99926

Figure 5.28 FTIR spectra in the 1800-1500 cm^{-1} range for SP12%TG

Hemp Protein

For hemp protein based systems, both the control (Figures 5.29 - 5.30 - 5.31) and treated (Figures 5.32- 5.33 - 5.34) samples, also show the amide I band centred around 1650 cm^{-1}, exhibiting an asymmetrical shape and the amide II band centred around 1520 cm^{-1}. HP6% shows a β-sheet conformation predominant peak around 1637 cm^{-1}, β-turns at 1605, 1662 cm^{-1} and 1680 cm^{-1} (Figure 5.29).

Increasing the protein content, in HP9%, (Figure 5.30) reduces the overall amide I band signal with a reduction in intensity for the peaks assigned to β-turns and β-sheets. In the HP9%

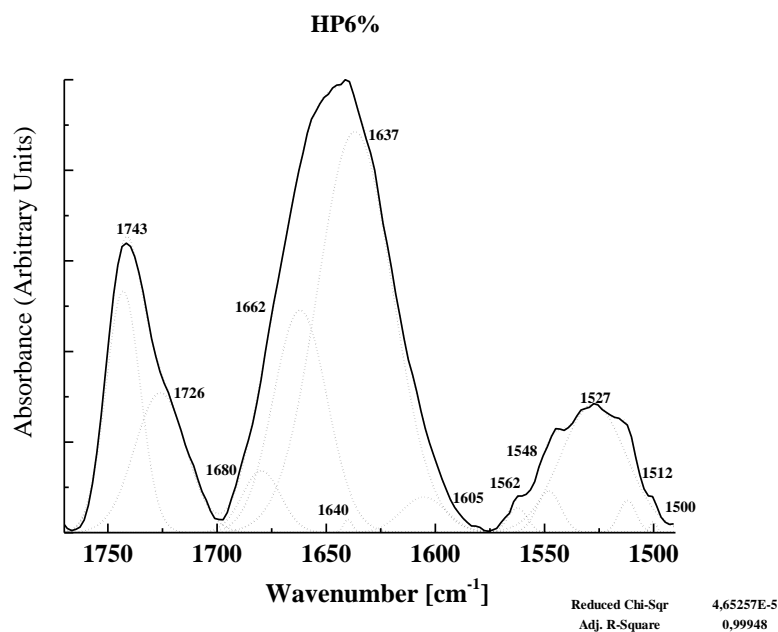sample, the band around 1618 cm^{-1} could be associated with the formation of intermolecular β-sheets.

HP6%

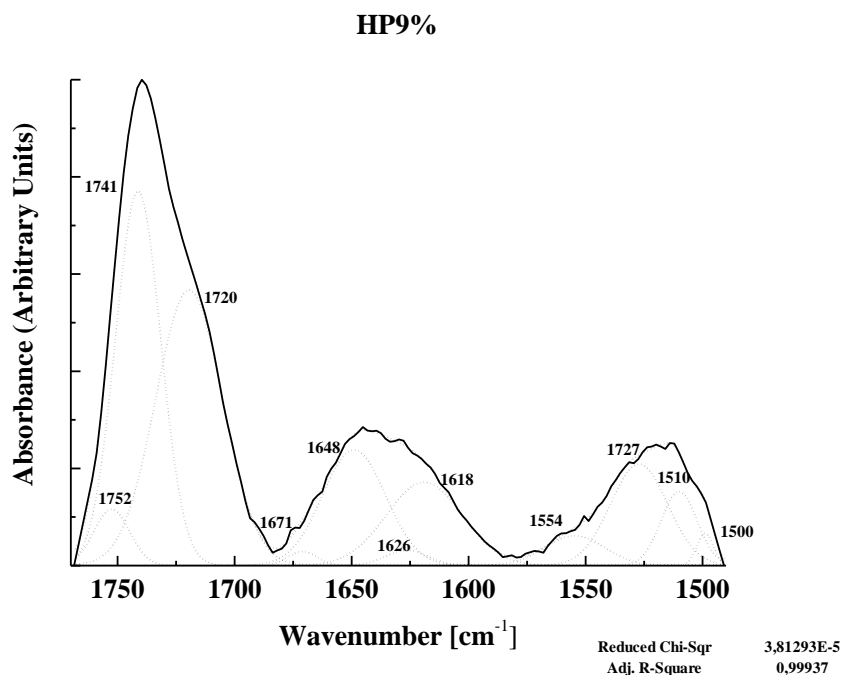*Figure 5.29 FTIR spectra in the 1800-1500 cm^{-1} range for HP6%*

HP9%

Figure 5.30. FTIR spectra in the 1800-1500 cm^{-1} range for) HP9%

The band previously identified at wavenumbers usually associated with the formation of intermolecular β-sheets, that could still be slightly shifted to 1612 cm^{-1} for HP12%, seems to show a reduced intensity when the amount of protein is increased up to 12%. At the higher hemp protein concentration, a splitting of the peak in the amide I region can also be observed (Figure 5.31).

HP12%

Reduced Chi-Sqr 1,32463E-4
Adj. R-Square 0,99789
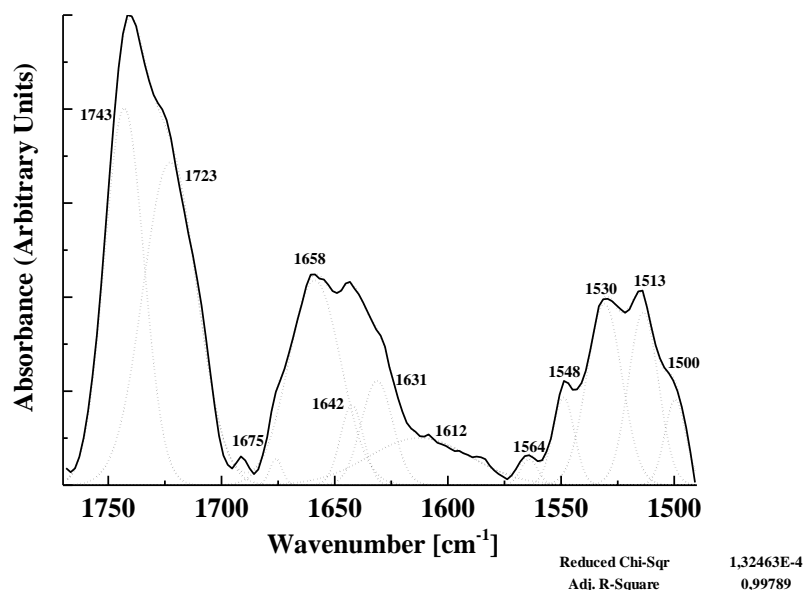
Figure 5.31   FTIR spectra in the 1800-1500 cm^{-1} range for HP12%

The presence of the enzyme reduced β-turn and β-sheet conformation are weak at low protein concentration (HP6%TG) and increase at 12%. For the HP9%TG sample, a broad peak can be observed at 1635 cm^{-1} (β-sheet weekly H-bonded), while other, weaker sub-peaks, due to β-turns (1664 cm^{-1}) α-helices (1648 cm^{-1}), and β-sheets (1634 cm^{-1}), can also be observed in the amide I region for HP9%TG.

HP6%TG

Reduced Chi-Sqr 1,67939E-5
Adj. R-Square 0,9997
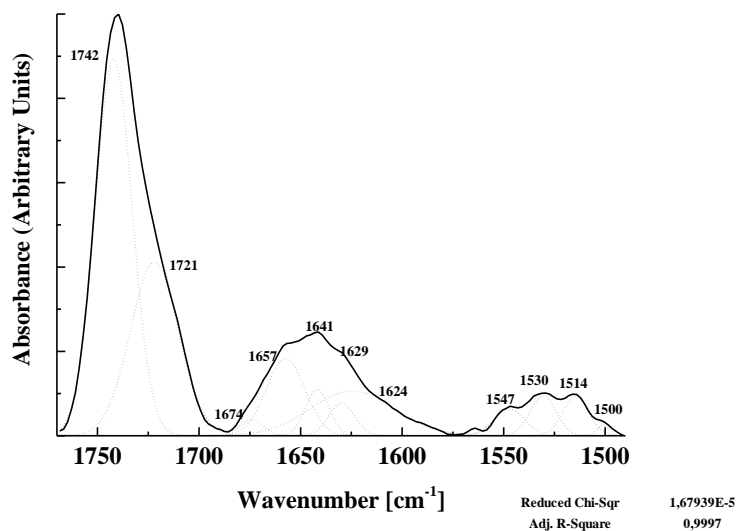
Figure 5.32. FTIR spectra in the 1800-1500 cm^{-1} range for HP6%TG

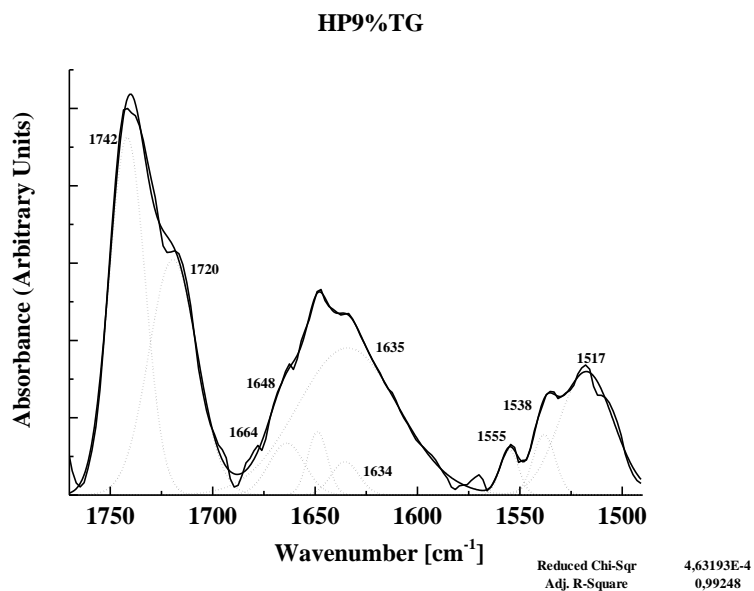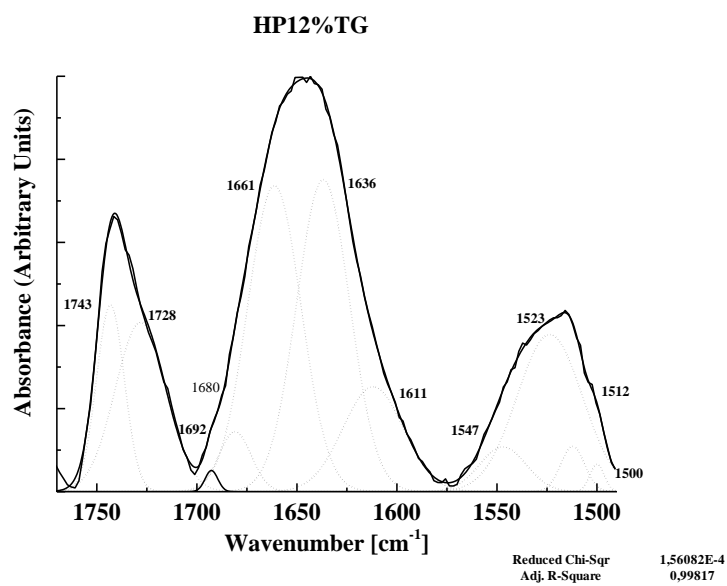*Figure FTIR 5.33 Spectra in the 1800-1500 cm⁻¹ range for HP9%TG*

Figure 5.34 FTIR spectra in the 1800-1500 cm⁻¹ range for HP12%TG

5.3.5 Porosimetry

BET analysis, both on raw materials and breadcrumbs, typically gave a Type II isotherm (Figure 5.35 is reported below as an example for the resistant starch used in the studied materials).
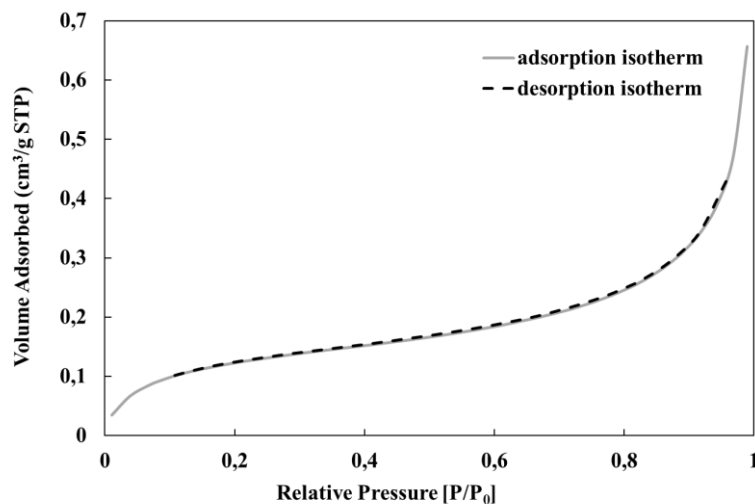

Figure 5.35 Adsorption *(solid line) and desorption (dashed line) isotherms of nitrogen for resistant starch*

The volume of adsorbed gas is very small in all cases, when compared to porous materials that are usually analysed using this technique. Results obtained, in terms of specific area, which has been evaluated according to the procedure explained in Chapter 3, Section 3.2.2.3, are shown below (Figures 5.36 and 5.37). It has not been possible to obtain results for all samples and, in particular, after repeated measurements for HP9%, HP6% TG, HP9% TG, and HP12% TG, the measurements were not completed or gave nonsensical results. In general, what emerges from Figures 5.36-5.37 is that in both cases for samples prepared with soy and hemp proteins, the specific surface is smaller than that of the RS control sample and this is the only significant difference found by comparing the analysed samples.
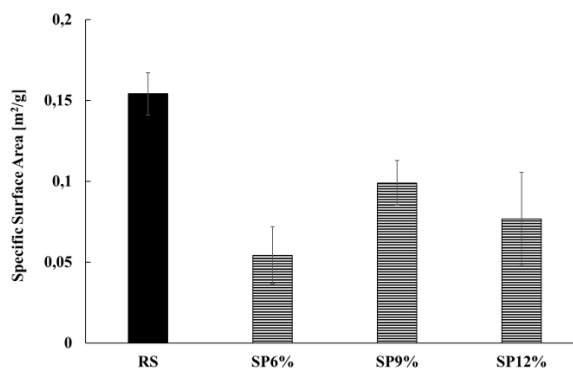

Figure 5.36 SBET for samples prepared adding soy proteins at different level
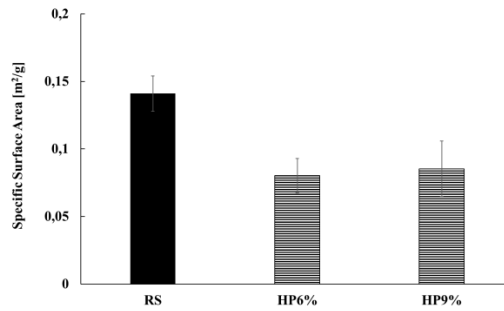
Figure 5.37 S_{BET} for samples prepared adding hemp proteins at two different levels

No significant differences can be observed when the protein concentration is varied or by adding the enzyme mTG-ase to the samples prepared with soy protein so that it was possible to complete the measurements (Figure 5.38).
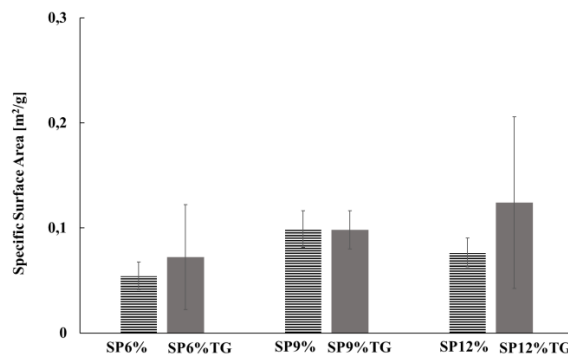

Figure 5.38 Comparison of S_{BET} values for samples prepared adding soy proteins and m-TGase

5.3.6 SEM analysis

The sample which performed best in terms of both rheological analysis and baking performance, was subjected to scanning electronic microscopy, to qualitatively explore its surface microstructure. The protein that gave the best results was hemp, and the images were obtained for both the HP9% sample and the HP9% TG one treated with the enzyme. An image of the RS control dough was also collected for reference (Figure 5.39).
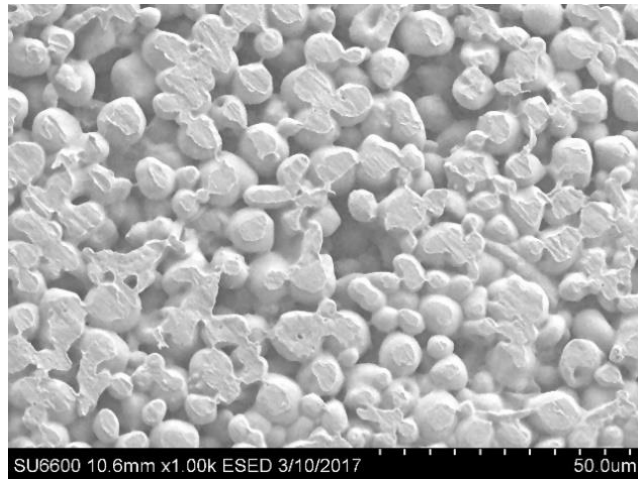
Figure 5.39 Scanning electron microscopy inside of the gluten-free bread crumb for RS

From the control image (Figure 5.39), the sample appears as a simple aggregation of spheroidal particles. These particles are starch granules swollen because of the gelatinisation. Introducing a certain amount of protein, the formation of a more irregular surface is observed and, form Figure 5.40, filament aggregates, which may be attributed to the formed protein network, can also be observed.
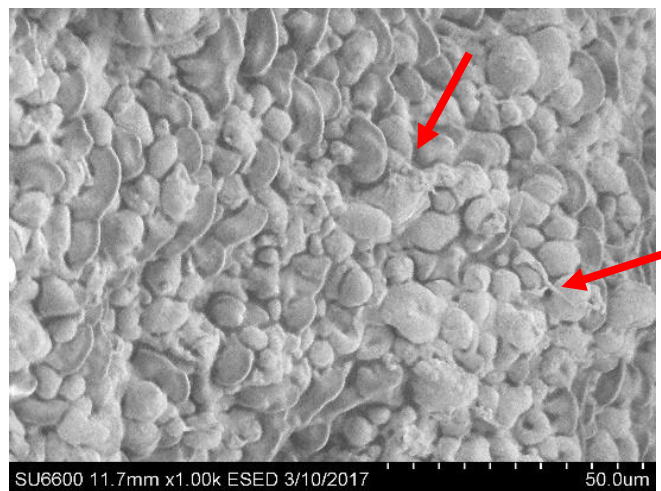

Figure 5.4 Scanning electron microscopy inside of the gluten-free bread crumb for HP9%

By adding the enzyme to the sample at 9% hemp protein, while observing a similar morphology, the aggregate strands found in the untreated sample are not observed in the treated one (Figure 5.41 a and b). This result should be considered in accordance with that found from rheological analysis and visual inspection of the cooked bread; the transglutaminase enzyme does not, in fact, yield good results in terms of structuring, when used with hemp proteins.
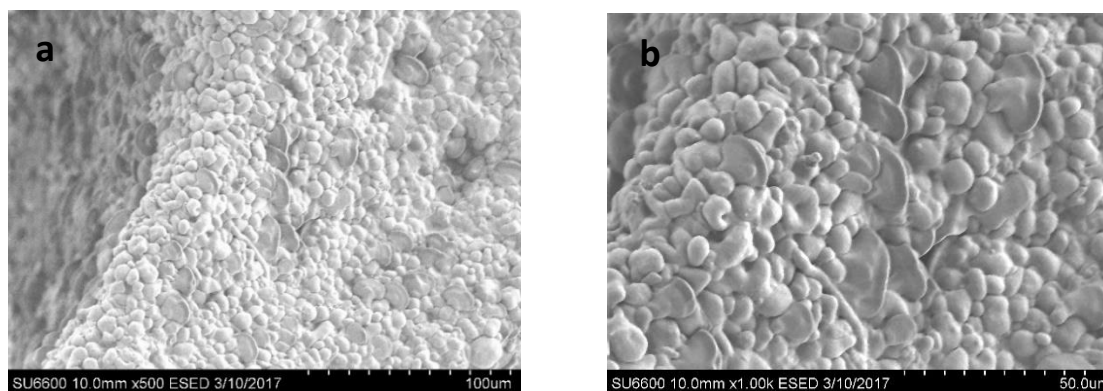
Figure 5.41 Scanning electron microscopy inside of the gluten-free bread crumb for HP9%TG at two different magnification

Conclusions

In this chapter the physico-chemical characteristics of GF bread model systems, prepared with resistant starch and vegetable proteins, were investigated, with the aim of deepening the knowledge of these alternative GF ingredients, which are becoming more popular in the GF industry thanks to their great technological properties and their known benefits to consumer health.

Thermal analysis showed that, for these model systems, the type of protein used (soy and hemp), which are very different from gluten proteins, are not able to strongly modify water distribution in the bread matrix. The soy protein content did not lead to relevant results in terms of water distribution, while in the case of hemp small variations were observed and, in particular, an increase in the protein content produced an increase in the fraction of the more tightly bound water. mTG-ase modified the water distribution and T_{peak} only in the case of soy protein.

FT-IR analysis showed that, for both soy and hemp proteins, changing the amount of protein, as well as adding a crosslinking enzyme, caused changes in the bread gluten conformations related to amide I and amide II bands, with a more evident effect on the intensity of the amide I band, which is directly related to the protein backbone conformation. High soy protein concentrations led to dominant β-turns, and β-sheet conformations. For the treated bread, the enzyme reduced the α-helix signal intensity. At higher protein contents, the soy treated bread gained a β-sheet predominant conformation at the expense of β-turns. The presence of the enzyme, in the hemp treated bread, at the lowest protein concentration, reduced β-turn and β-sheet conformation signals and increased them at the higher hemp protein concentration.

The results indicate that significant reactive and conformational processes occur within the dough matrix during processing, which are highly dependent on composition.

References

- Barth, A. (2007) Infrared spectroscopy of proteins. Biochimica Et Biophysica Acta-Bioenergetics, 1767, 1073-1101.

- Cai, S. & B. Singh (1999) Identification of beta-turn and random coil amide III infrared bands for secondary structure estimation of proteins. *Biophysical Chemistry*, 80, 7-20.

- Cerna, M., A. Barros, A. Nunes, S. Rocha, I. Delgadillo, J. Copikova & M. Coimbra (2003) Use of FT-IR spectroscopy as a tool for the analysis of polysaccharide food additives. *Carbohydrate Polymers*, 51, 383-389.

- Chirgadze, Y. N., & Nevskaya, N. A. (1976). Infrared spectra and resonance interaction of amide-I vibration of the antiparallel-chain pleated sheet. *Biopolymers*, 15(4), 607-625.

- Dorado, J., G. Almendros, J. Field & R. Sierra-Alvarez (2001) Infrared spectroscopy analysis of hemp (Cannabis sativa) after selective delignification by Bjerkandera sp at different nitrogen levels. *Enzyme and Microbial Technology*, 28, 550-559.

- Fessas, D., Schiraldi, A. (2001). Water properties in wheat flour dough I: classical thermogravimetry approach. *Food Chemistry*. 72, 237-244.143

- Fessas, D., Signorelli, M., Pagani, A., Mariotti, M., Iametti, S., & Schiraldi, A. (2008). Guidelines for buckwheat enriched bread: thermal analysis approach. *Journal of Thermal Analysis and Calorimetry*, 91(1), 9-16.

- Flores-Morales, A., M. Jimenez-Estrada & R. Mora-Escobedo (2012) Determination of the structural changes by FT-IR, Raman, and CP/MAS C-13 NMR spectroscopy on retrograded starch of maize tortillas. *Carbohydrate Polymers*, 87, 61-68.

- Garside, P. & P. Wyeth (2003) Identification of cellulosic fibres by FTIR spectroscopy - Thread and single fibre analysis by attenuated total reflectance. *Studies in Conservation*, 48, 269-275.

- Hammann, F. & M. Schmid (2014) Determination and Quantification of Molecular Interactions in Protein Films: A Review. Materials, 7, 7975-7996.

- Jyothi, A., S. Moorthy & K. Rajasekharan (2006) Effect of cross-linking with epichlorohydrin on the properties of cassava (Manihot esculenta Crantz) starch. *Starch-Starke*, 58, 292-299.

- Kacurakova, M. & R. Wilson (2001) Developments in mid-infrared FT-IR spectroscopy of selected carbohydrates. *Carbohydrate Polymers*, 44, 291-303.

- Kacurakova, M., P. Capek, V. Sasinkova, N. Wellner & A. Ebringerova (2000) FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses. *Carbohydrate Polymers*, 43, 195-203.

- Kizil, R., J. Irudayaraj & K. Seetharaman (2002) Characterization of irradiated starches by using FT-Raman and FTIR spectroscopy. *Journal of Agricultural and Food Chemistry*, 50, 3912-3918.

- Kong, J. & S. Yu (2007) Fourier transform infrared spectroscopic analysis of protein secondary structures. Acta Biochimica Et Biophysica Sinica, 39, 549-559.

- Krimm, S. & J. Bandekar (1986) Vibrational Spectroscopy and Conformation Of Peptides, Polypeptides, And Proteins. *Advances in Protein Chemistry*, 38, 181-364.

- Liu, Q., G. Charlet, S. Yelle & J. Arul (2002) Phase transition in potato starch-water system I. Starch gelatinization at high moisture level. *Food Research International*, 35, 397-407.

- Madamba, P. S., Driscoll, R. H., & Buckle, K. A. (1996). The thin-layer drying characteristics of garlic slices. *Journal of food engineering*, *29*(1), 75-97.

- Matheus, S., Friess, W., & Mahler, H. C. (2006). FTIR and nDSC as analytical tools for high-concentration protein formulations. *Pharmaceutical research*, 23(6), 1350-1363.

- Mejia, C., L. Mauer & B. Hamaker (2007) Similarities and differences in secondary structure of viscoelastic polymers of maize alpha-zein and wheat gluten proteins. *Journal of Cereal Science*, 45, 353-359.

- Pavlovic, S. & P. Brandao (2003) Adsorption of starch, amylose, amylopectin and glucose monomer and their effect on the flotation of hematite and quartz. *Minerals Engineering*, 16, 1117-1122.

- Robertson, G., K. Gregorski & T. Cao (2006) Changes in secondary protein structures during mixing development of high absorption (90%) flour and water mixtures. *Cereal Chemistry*, 83, 136-142.

- Servaty, R., Schiller, J., Binder, H., & Arnold, K. (2001). Hydration of polymeric components of cartilage—an infrared spectroscopic study on hyaluronic acid and chondroitin sulphate. *International Journal of Biological Macromolecules*, 28(2), 121-127.

- Sevenou, O., Hill, S. E., Farhat, I. A., & Mitchell, J. R. (2002). Organisation of the external region of the starch granule as determined by infrared spectroscopy. International *Journal of Biological Macromolecules*, 31(1), 79-85.

- Sinelli, N., E. Casiraghi & G. Downey (2008) Studies on proofing of yeasted bread dough using near- and mid-infrared spectroscopy. *Journal of Agricultural and Food Chemistry*, 56, 922-931.

- Sivam, A., D. Sun-Waterhouse, C. Perera & G. Waterhouse (2013) Application of FT-IR and Raman spectroscopy for the study of biopolymers in breads fortified with fibre and polyphenols. *Food Research International*, 50, 574-585.

- Tori, H. & M. Tasumi (1992) Model-Calculation On the Amide-I Ifrared Bands Of Globular-Proteins. *Journal of Chemical Physics*, 96, 3379-3387.

- van Velzen, E., J. van Duynhoven, P. Pudney, P. Weegels & J. van der Maas (2003) Factors associated with dough stickiness as sensed by attenuated total reflectance infrared spectroscopy. *Cereal Chemistry*, 80, 378-382.

- vanSoest, J., H. Tournois, D. deWit & J. Vliegenthart (1995) Short-range structure in (partially) crystalline potato starch determined with attenuated total reflectance Fourier-transform IR spectroscopy. *Carbohydrate Research*, 279, 201-214.

- Vasko, P. D., Blackwell, J., & Koenig, J. L. (1972). Infrared and Raman spectroscopy of carbohydrates.: Part II: Normal coordinate analysis of α-D-glucose. *Carbohydrate Research*, 23(3), 407-416.

General conclusions

In this work new alternatives ingredients were used in the formulation of LP/GF baked goods with particular attention given to gluten-free bread.

By exploring how food molecules interact through the study of their chemical, mechanical and thermal properties, the aim of the project was to improve the GF bread quality from a nutritional, and texture point of view, enhancing the fiber content, adjusting the protein type and amount in the formulations, aiming to reduce the ingredients, benefitting taste and costs alike.

The study of this type of system was carried out in two steps.

In the first part, after a bibliographic research and a preliminary study on GF foods, two types of flours were chosen, for their capabilities in terms of baking performance and their nutritional characteristics which can bring health benefits to consumers.

The product design of *GF breads* enriched with fibers was performed starting from a bread recipe and substituting the whole amount of flour with a mixture of the chosen GF ones, also adding cellulose derivatives hydrocolloids and studying the effect of the water amount.

Performing rheological, thermal and spectroscopical analyses it has been possible to demonstrate that the use of the proper hydrocolloid concentration, with increased water level, can lead to GF doughs more similar to the standard wheat ones without the addition of excessive ingredients. In fact, the textural aspect of the GF bread improved simply replacing the amount of wheat with an optimized blend of buckwheat and rice flour and the addition of cellulose derived hydrocolloids, which, besides the role of structuring agents, also belong the dietary fibre class. The obtained final product has a fiber content of 4% (w/w).

Despite the excellent results achieved in this first part, the final product results in relatively high caloric bread (about 440Kcal / 100g). This is due to the type of starch naturally contained in the used flours, for this reason, the second part of the work focused more on the issues related to the caloric aspect, with the aim of designing a *GF flour* from the controlled properties, using the information gained from the first part about the amount of water more suitable for these systems, which is higher if compared to the one used in the standard products, and starting from a specific type of starch which is different from the traditional ones. A resistant starch was chosen as a starting point for the research. This product acts like a fiber, because of its high content in amylose and it helps to keep low the glucose level in blood and brings other significant health benefits. Starch by itself is not capable of generating an aerated structure when used alone in replacement of wheat flour, for this reason it has been chosen to gradually

rate it in vegetable proteins instead of animals ones, to test their structuring abilities. A microbial enzyme was added too with the aim of improving the proteins crosslink. This enzyme is widely used in the meat and fish industry, but it seems that can work also with some types of vegetable proteins, depending on the aminoacyls sequence and exposition.

In this second part, two types of proteins were tested: the soy proteins, known and used in the GF food industries, and the hemp proteins. The latest are not yet widely used in the production of GF bread, but they exhibited excellent abilities in producing honeycombed matrices in food systems, showing structuring properties better than the soy even if the obtained model systems still needs to be improved. Some of the GF bread model systems obtained in this second part, are very similar to traditional bread, and thanks to the combined use of resistant starch and vegetable proteins it is possible to obtain a product with less calories (330 Kcal / 100g).

As a general conclusion, rheology with other research techniques have proved to be useful as tool for studying these complex food systems and improve them. In fact, nowadays a lot of aspect needs to be improved and the different approaches used in this study have highlighted that the most important aspect have to be considered in this type of systems is that their macroscopical characteristics are strongly linked to the temperature and still a very little literature exist about this.